

**ROLE OF MMP2, MMP3 AND MMP9 IN THE DEVELOPMENT OF  
BREAST CANCER BRAIN AND LUNG METASTASIS IN A  
SYNGENEIC RAT MODEL**

A Dissertation

by

ODETE RODRIGUES MENDES

Submitted to the Office of Graduate Studies of  
Texas A&M University  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2005

Major Subject: Veterinary Pathology

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## ABSTRACT

Role of MMP2, MMP3 and MMP9 in the Development of Breast Cancer Brain  
and Lung Metastasis in a Syngeneic Rat Model.

(August 2005)

Odete Rodrigues Mendes,

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In order to study the expression of MMP2, MMP 3 and MMP9 in breast cancer brain and lung metastasis, we used a syngeneic rat model of distant metastasis of ENU1564, a carcinogen-induced mammary adenocarcinoma cell line. At six weeks post inoculation we observed development of micro-metastasis in the brain and lung. Immunohistochemistry and Western blotting analyses showed that MMP 2, -3 and -9 protein expression is consistently significantly higher in neoplastic brain tissue compared to normal brain tissue. Lung metastases express abundant MMP2, -3 and -9 in neoplastic cell cytoplasm. *In situ* zymography revealed gelatinase activity within the brain metastasis. Gel zymography showed an increase in MMP2 and MMP3 activity in brain metastasis. Furthermore, we were able to significantly decrease the development of breast cancer brain and lung metastasis in animals by treatment with PD 166793, a selective synthetic MMP inhibitor. In addition, PD 166793 decreased the *in vitro* invasive cell behavior of ENU1546. TIMP2 overexpression also decreased the development of breast cancer lung metastasis in our model. Our results suggest that MMP2, -3 and -9 may be

involved in the process of metastasis of breast cancer to the brain and lung.

Because astrocytes have been associated with breast cancer brain metastasis we evaluated the role of astrocytes and ERK2 pathway in MMP2 up-regulation in BC brain metastasis. A significant decrease in brain metastases development, and orthotopic tumor size and weight were observed in animals inoculated with ENU1564-TIMP2 cells. These were associated with decreased MMP2 activity, as demonstrated by gel zymography. Rat astrocyte-conditioned media increased expression of MMP2 in ENU1564 cells and increased *in vitro* cell invasion of ENU1564 and ENU1564-TIMP2 cells. Blockage of ERK1/2 phosphorylation by treatment with PD98059 decreased the expression of MMP2 in cancer cells grown in rat astrocyte-conditioned media. We determine that MMP2 plays a role in *in vivo* development of breast cancer brain metastases. Additionally, we conclude that astrocytes are associated with expression of MMP2 in cancer cells via ERK1/2 signaling pathway.

## DEDICATION

To Paula

## **ACKNOWLEDGMENTS**

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# CHAPTER I

## INTRODUCTION

### **Overview on matrix metalloproteinases**

Matrix metalloproteinases (MMP) are structurally related endopeptidases that have multiple biological roles including the degradation of the extracellular matrix (ECM). Physiologically these enzymes play a role in normal tissue remodeling as well as in angiogenesis and mammary gland involution. They belong to a family of 23 gene products, which encode for zinc-dependent and calcium dependent proteases that are endopeptidases [1]. There are also two other large families of proteases that have major roles in extracellular proteolysis, the ADAM family (a disintegrin and metalloproteinase domain, with about 33 members in humans) and the ADAMTS family (a disintegrin-like and metalloproteinase domain (reprolysin type) with thrombospondin type I repeats, with about 19 members in humans).

Numerous classifications of MMPs can be made. Based on their solubility they can be divided in two major groups.

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This dissertation follows the style of Clinical & Experimental Metastasis.

(1) Soluble type MMPs; include collagenases, stromelysins, gelatinases and matrilysins. (2) Membrane-anchored metalloproteinases; include Type II and type II types.

They can also be classified according to their substrates. They are known to degrade a large array of substrates such as Collagens (C), Fibronectin (FN), Cartilage oligomeric protein (COMP), Laminin (LN) and Proteoglycan (PG) [2] (**Table 1**).

MMP regulation occurs at multiple levels that include transcription, activation of zymogen forms, and activity of extracellular inhibitors [1]. There is moderate variation in the protein structure of MMPs. Metalloproteinases are composed by a pre-catalytic, a pro-catalytic domain, a fibronectin-like domain, a domain for binding to zinc and a homeopexin domain (**Figure 1**).



Figure 1. Domain structure of MMP proteins.

These enzymes depend on zinc for catalytic activity [3]. The presence of the pro-domain keeps the enzyme inactive. In order to be activated a cysteine residue that inactivates ligand binding to the zinc catalytic site must be removed. This can be done by

conformational change or proteolysis accomplished by plasmin or other MMPs. MMP zymogens can be activated by themselves; for instance MT1-MMP activates MMP2 and requires TIMP2 binding to its active site in order to do so [4]. Inactivation of MMPs can occur by direct interaction with tissue inhibitors of MMPs (TIMPs), alpha 2 macroglobulin and other molecules such as pro-collagen C-proteinase enhancer [5].

MMPs are responsible for the turnover of the ECM that is rich in growth factors and other bioactive molecules and in this way contribute to numerous physiological and pathologic processes. Matrix metalloproteinases have multiple important roles in cancer development.

- (1) MMPs cause tumor cell initiation and growth. MMP3 has pre-neoplastic activity and is correlated in cancer cell malignant phenotype.
- (2) MMPs are crucial in degradation of basement membrane and extra cellular matrix that are fundamental for cancer cell invasion, and metastasis establishment **(Figure 2)**.
- (3) MMPs are related to tumor angiogenesis. MMP2 is responsible for laminin-5 degradation.
- (4) MMPs are associated with breast physiological development in which MMP9 stimulates increased cell proliferation, branching and morphogenesis by  $\text{TNF}\alpha$  [7].
- (5) MMP9 is also important for cancer cell migration and it cooperates with  $\alpha\text{v}\beta 3$  integrin [8].

**Table 1.** MMP classification.

<b>Protein</b>	<b>MMP</b>	<b>MW (kDa)</b>	<b>Substrate(s)</b>
<b>Soluble Type</b>			
<b>Collagenase</b>			
Collagenase 1	MMP1	52/41	C-II, II, III, X
Collagenase 2	MMP8	5/64	C-I, II, III
Collagenase 3	MMP13	65/55	C-I, II, IV, X, XIV, FN, aggrecan, tenascin
<b>Stromelysin</b>			
Stromelysin 1	MMP3	57/45,28	PG, FN, C-III, IV, VII, IX, Gelatin, LN
Stromelysin 2	MMP10	56/47,24	C-III, IV, V, gelatin, PG, FN
<b>Gelatinase</b>			
Gelatinase A	MMP2	72/67	Gelatin, C-IV, FN, PG, LN
Gelatinase B	MMP9	92/67	Gelatin, C-III, IV, V, elastin
<b>Matrylisin</b>			
Matrylisin 1	MMP7	28/19	Gelatin, C-IV, FN, PG, LN
Matrylisin 2	MMP26	29/19	Gelatin, C-IV, FN, fibrinogen
<b>Others</b>			
Stromelysin 3	MMP11	58/28	Gelatin, PG, LN, FN
Epilysin	MMP28	56/45	Casein
Not named	MMP19	57	Gelatin, aggrecan, COMP, LN, nidogen, tenascin, C-IV, FN
Matalloelastase	MMP12	54/45,22	Elastin
Enamelysin	MMP20	54/43	Amelogenin, aggrecan, COMP, FN, CIV, LN
<b><u>Membrane-anchored</u></b>			
<b>Type I transmembrane-type</b>			
MT1-MMP	MMP14	66/60	C I, II, III, gelatin, PG, FN
MT2-MMP	MMP15	68/62	FN, aggrecan, nidogen, tenascin, perlecan, LN
MT3-MMP	MMP16	64/55	C-III, FN, gelatin
MT5-MMP	MMP24	73/64	PG
<b>GPI-type</b>			
MT4-MMP	MMP14	71/67	Fibrin, fibrinogen
MT6-MMP	MMP25	62/58	Gelatin
<b>Type II transmembrane-type</b>			
Cystine-array-MMP	MMP23	66	Gelatin



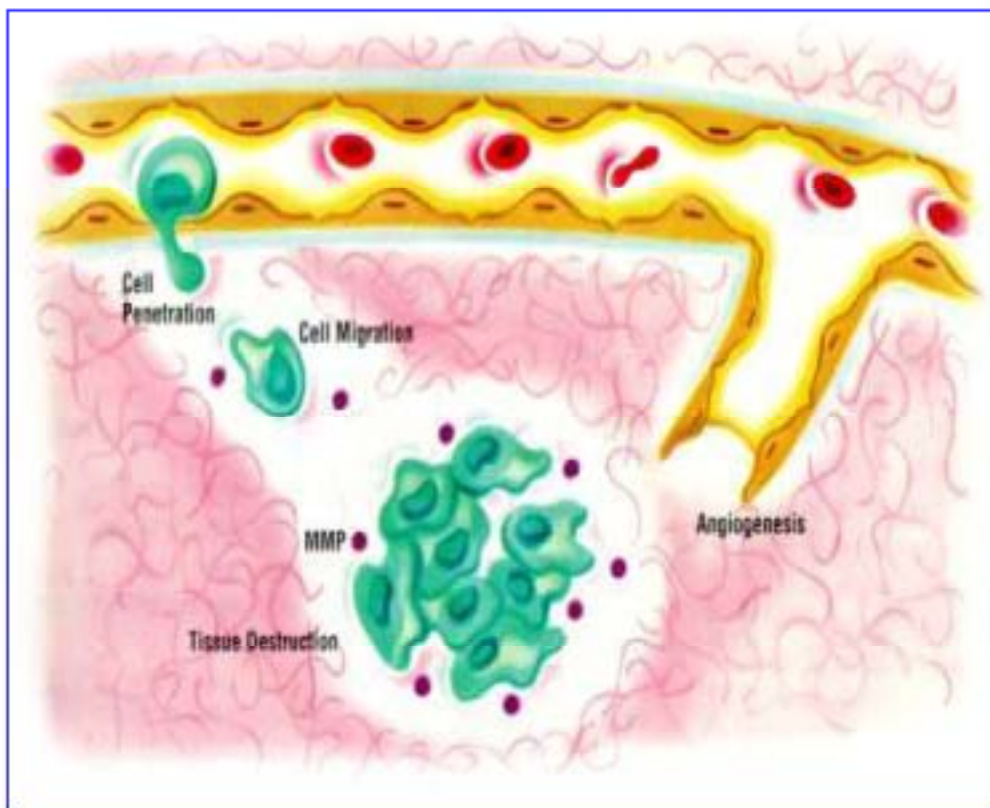


Figure 2. MMPs role in cancer cell extravasation from blood vessel, cancer cell migration and establishment of metastatic foci.

(6) MMPs are correlated with cell proliferation. They release growth factors that are important for cancer and mammary gland cell multiplication.

(7) MMPs are important in apoptosis. They are reported to initiate apoptosis by causing loss of contact of cells to the basement membrane. TIMP1 and TIMP2 are thought to decrease apoptosis [3, 6].

Because of these and other functions MMPs are extensively correlated with breast cancer prognosis, cancer invasion and presence of metastasis. MMP2 and MMP9 are the two MMPs that are more frequently associated with breast cancer (BC) invasion and poor prognosis [6, 9, 10, 11, 12, 13, 14, 15, 16, 17,]. The role of these proteins is however in constant scrutiny and there are reports that contradict the association of MMPs in BC prognosis [12, 18].

### **Animal model**

Different animal models have been used for *in vivo* study of the role of MMPs in the development of cancer. Most of these studies describe lung, bone and/or node metastasis and are usually concurrent with the studies conducted on human patients that correlated MMPs with increased tumor invasion and metastatic behavior [10, 19, 20]. Several models have also been used to study metastatic disease. Most utilize immunocompromised animals, such as nude mice, that usually develop metastasis in the bone, lung and liver [21, 22]. These models do not consider the importance of the immune system in cancer development and its relevance to the development of metastatic disease. Transgenic models have also been used in the study of multiple cancer pathways [23, 24, 25, 26, 27]. We use a syngeneic model to study distant metastasis of breast cancer. The ENU1564 cell line used in our study is a highly metastatic breast cancer cell line originated from a N-ethyl-N-nitrosourea (ENU) induced mammary adenocarcinoma in a female Berlin-Druckrey IV (BD-IV) rat (**Figure 3**).

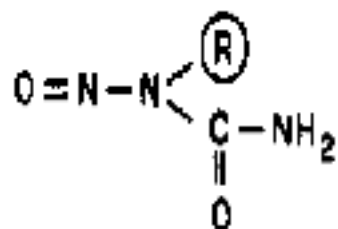


Figure 3. N-ethyl Nitrosurea (ENU) chemical structure.

ENU is a carcinogenic substance that acts as a specific alkylating agent of DNA and RNA nucleotide bases [28]. It has high affinity for oxygen causing frequent alkylations of the 2'-O ring in DNA phosphodiesteres and 2'-O of RNA riboses. [29]. All 2'-O from DNA bases can react with ENU and ring OH is also susceptible to ENU ethylation [30]. Additionally, ethylation can also occur in a ring N position. Ethylation of 7'-N causes rapid depurination and subsequent DNA chain breakage. Alkylation of 4'-O of uridine or thymidine causes mispairing. Alkylation of 2'-O causes depyrimidiazation and possible deletion [31]. In vivo the bases that are more susceptible to ethylation are thymidine and guanidine, followed by cytosine [32, 33, 34] (**Figure 4**).

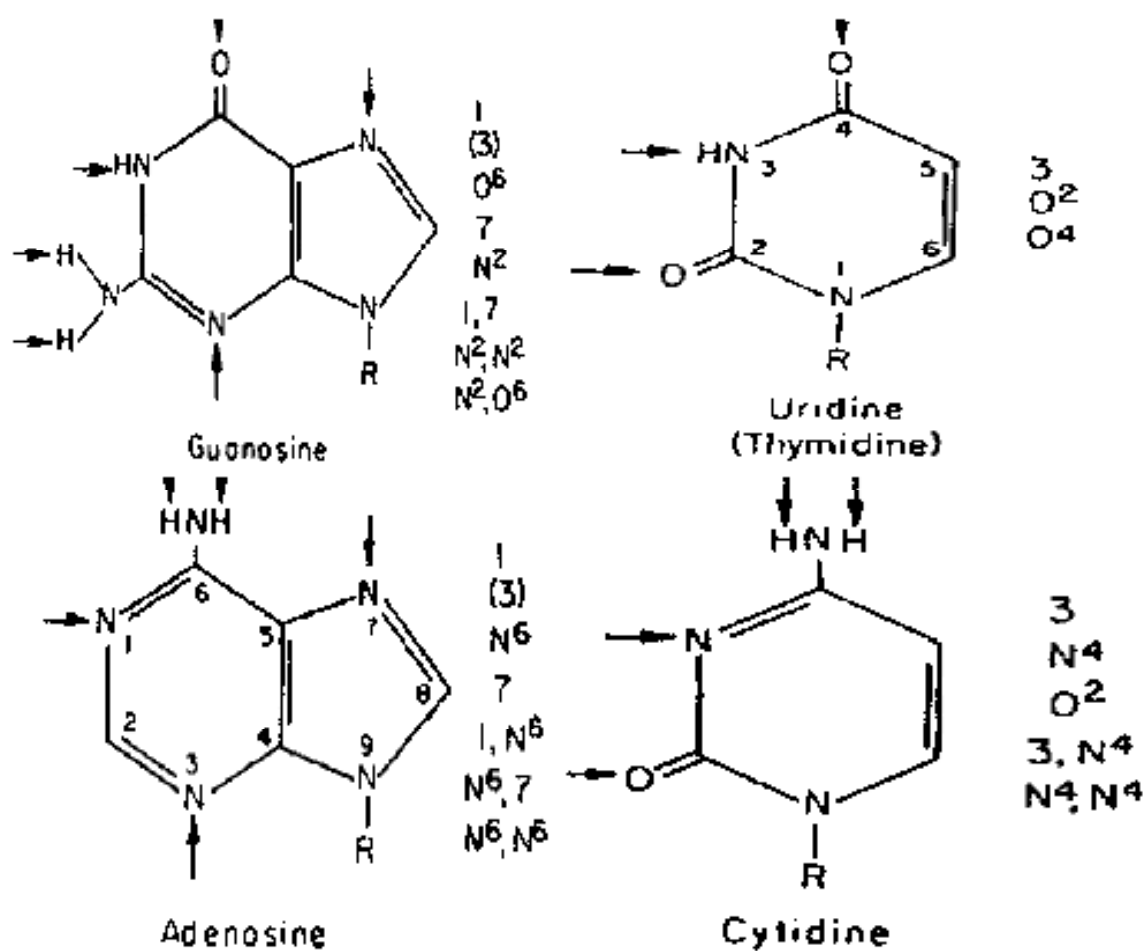


Figure 4. DNA and RNA base alkylation sites by N-ethyl Nitrosurea.

In studies described here syngeneic, 40 day old BD-IV rats are inoculated with  $1 \times 10^4$  ENU 1564 cells via left ventricle, they develop widespread colonization of breast cancer cells in numerous tissues such as bone, lung, kidney and brain (**Figure 5**).

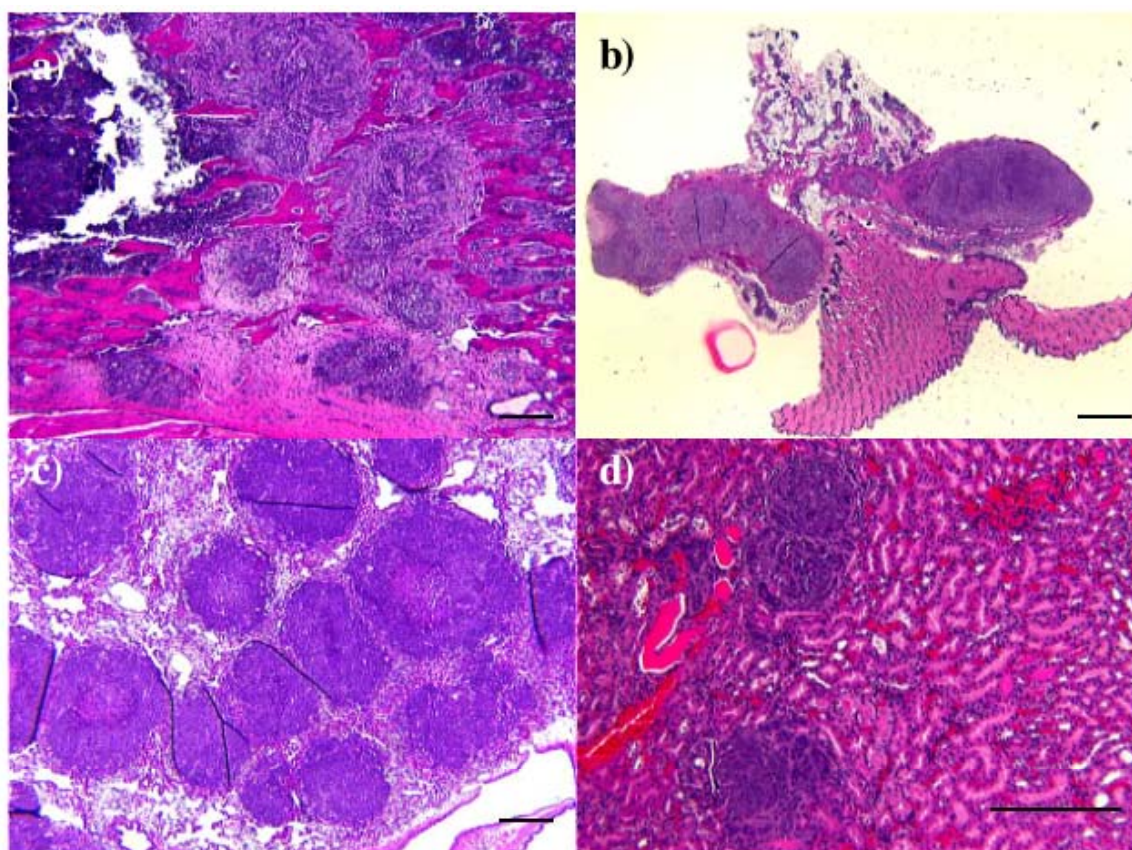


Figure 5. Histology of metastatic foci in ENU-BC IV rat metastatic model. a) Presence of metastatic tumor along long bone growth plate; b) mammary gland orthotopic tumor development; c) aspect of lung metastatic tumor; d) presence of kidney metastasis.

The metastasis cell morphology varies in different organs. Variations in the amount of necrosis, stroma and inflammation were also observed at different metastatic locations. The organ with the higher metastasis number is the lung. No metastases were identified in the digestive tube or liver (**Figure 6**).

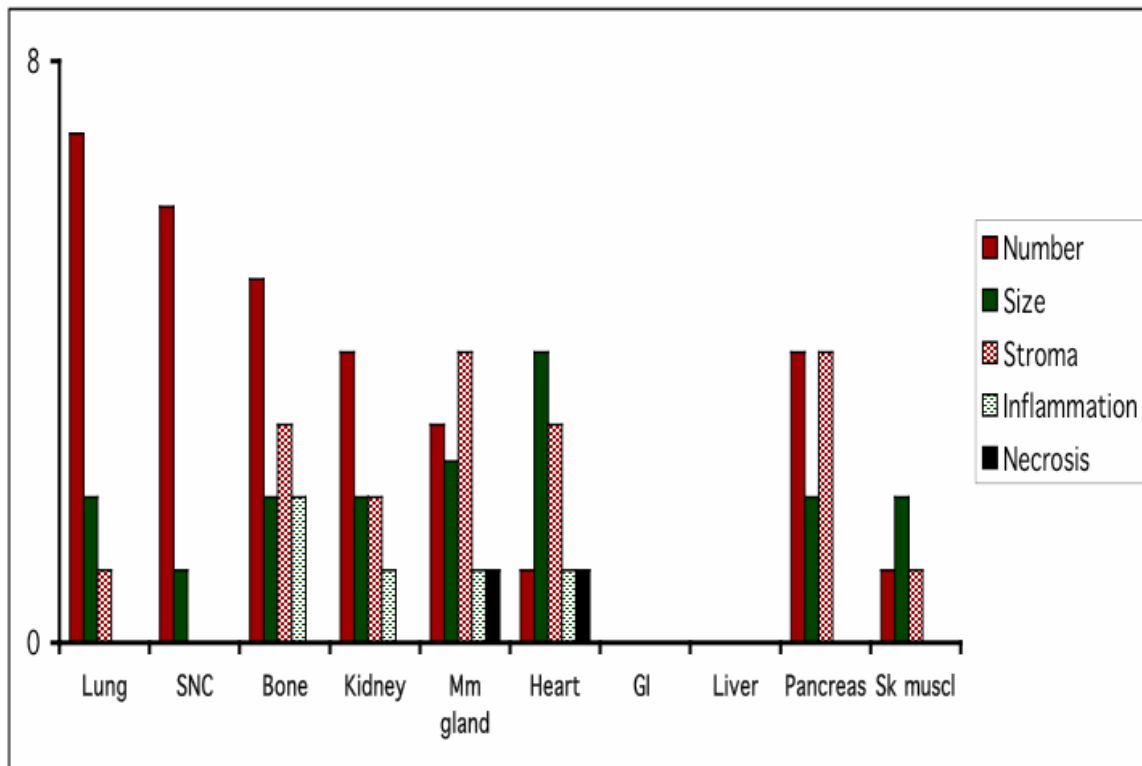


Figure 6. Characterization of tumor distribution in the different metastatic sites. Evaluation of histological characteristics including, tumor number, tumor size, amount of tumor stroma, presence of intratumoral inflammation and presence of intratumoral necrosis.

Since metastases were observed with regularity in the brain and bone (please see chapter II for distribution) our system was considered to be a reliable model for the study of distant breast cancer metastasis. In our experiments we also reproduced local mammary tumors by orthotopic inoculation of tumor cells in the inguinal mammary fat pad (**Figure 5**) resulting in local tumor growth of orthotopic tumor. Orthotopic tumors have multifocal

areas of necrosis, marked stromal desmoplastic response and moderate to marked inflammation (**Figure 7**).

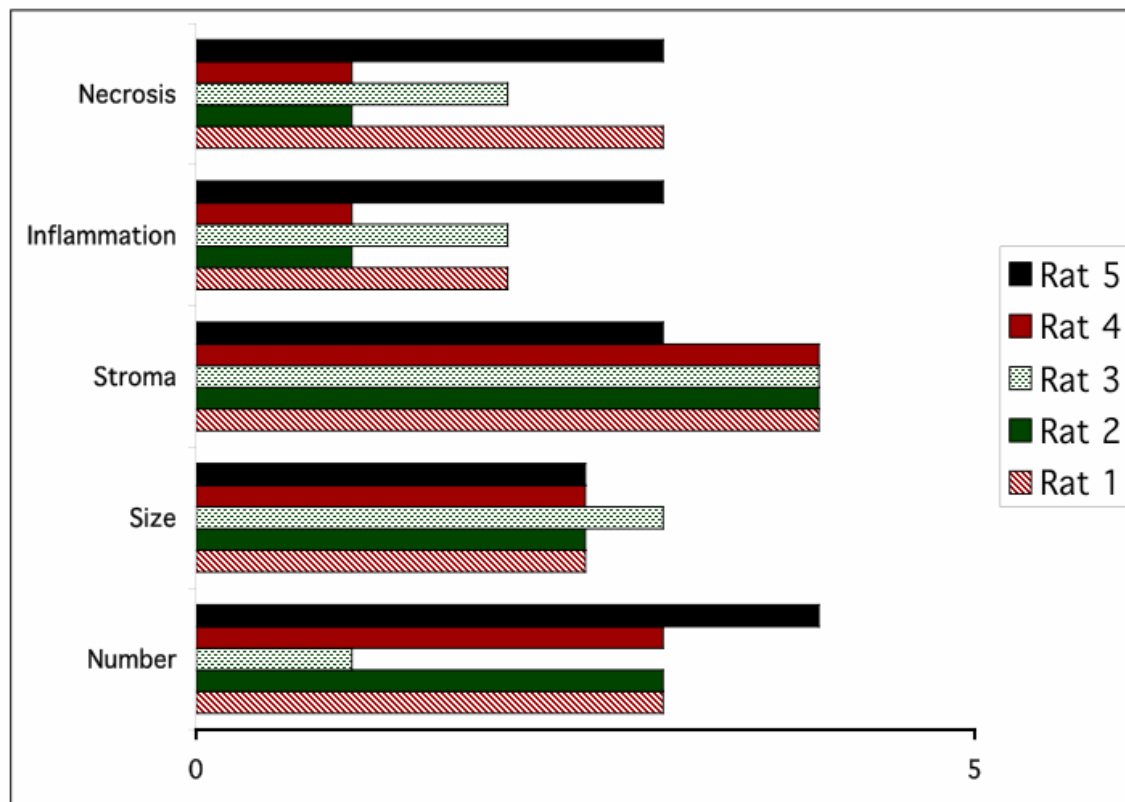


Figure 7. Evaluation of histological characteristics of mammary gland orthotopic tumor (including, tumor number, tumor size, amount of tumor stroma, presence of intratumoral inflammation and presence of intratumoral necrosis).

This is in contrast with the metastatic foci observed in the brain. Brain metastatic foci have absence of necrosis, inflammation and stromal response (**Figure 8**).

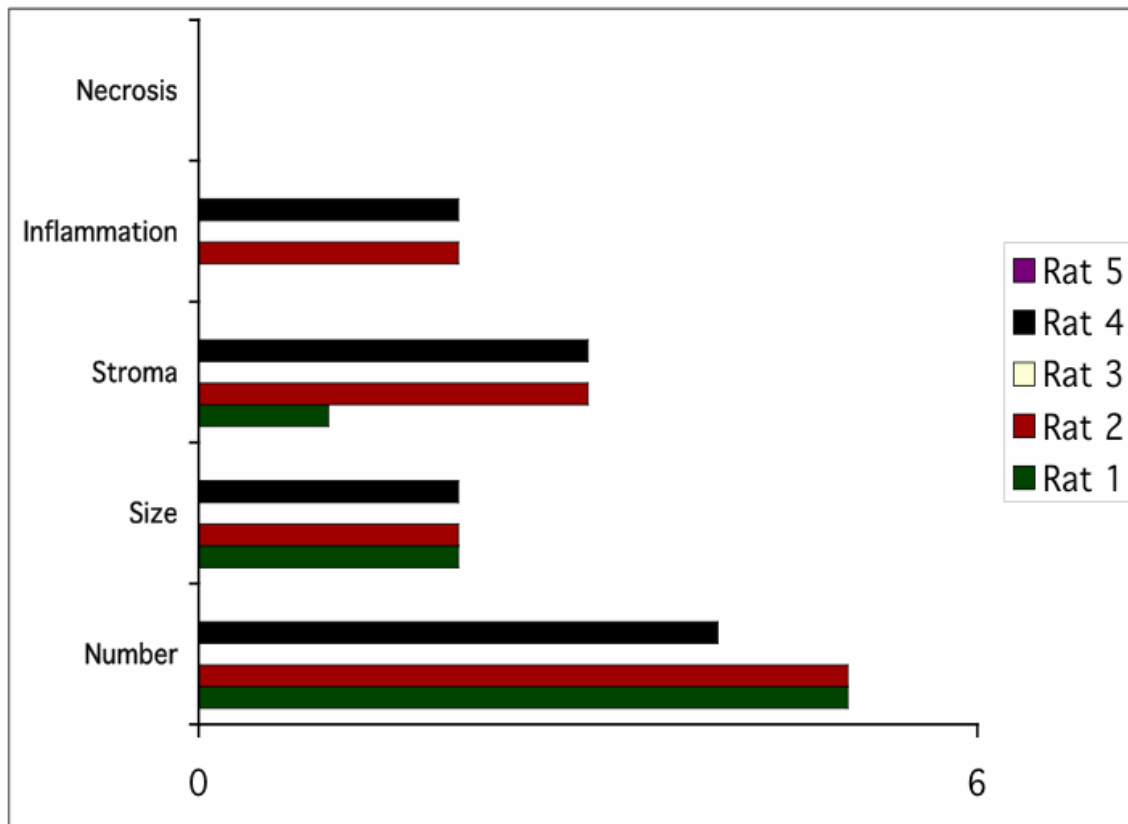


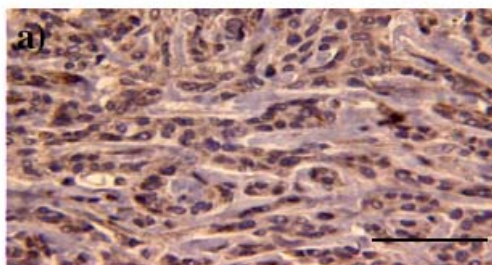
Figure 8. Evaluation of histological characteristics of brain metastatic tumor (including, tumor number, tumor size, amount of tumor stroma, presence of intratumoral inflammation and presence of intratumoral necrosis).

Our model was also considered to be appropriate to study MMP expression in breast cancer metastasis *in vivo*. Immunohistochemistry was performed on paraffin sections in order to determine whether neoplastic cells expressed MMPs. We evaluated

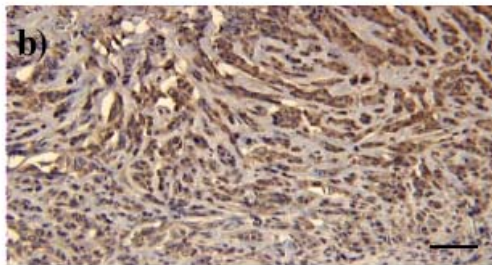


immunohistochemical staining for MMP2, -3 and -9 in all metastatic sites evaluated as well as in the orthotopic mammary tumor (**Figure 9**).

**MMP2**



**MMP3**



**MMP9**

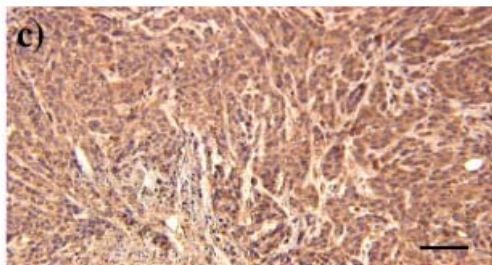


Figure 9. Immunohistochemical detection of MMP2, -3 and -9 in mammary gland orthotopic tumor.

### **MMP localization in tumors**

The morphological localization of MMPs intratumoral in breast cancer has been the subject of numerous studies. Different studies have sometime-contradictory data on the location of MMPs. Some co-localize MMP with neoplastic epithelial cells whereas others associated them with different components of the neoplastic stroma. Therefore, according to some reports, **MMP2** can be observed in stromal tumor fibroblasts and well-differentiated invasive cancer cells [35] in the neoplastic cell plasma membrane in peritumor stromal cells [36] and/or angiogenic blood vessels [37]. **MMP-9** has been associated with neoplastic cell plasma membrane, non-neoplastic ducts and acini, epithelial cells and macrophages, stromal fibroblasts and endothelial cells, tumor-infiltrating stromal cells, including neutrophils, macrophages, and vascular pericytes. Expression for **MMP-3** was observed both in tumor and stroma cells. Normal breast epithelia were weakly positive for MMP-3-mRNA [5]. Tumor cells and peritumor stroma showed low MMP-3 transcript levels, especially in medullary carcinomas. There is therefore a need to determine the exact location of MMPs in the different types of cells that compose the tumor in order to understand more about the way MMPs influence the invasive and metastatic process.

We observed staining for MMP2, -3 and -9 in the cytoplasm of neoplastic epithelial cells in all neoplastic sites evaluated (mammary gland, lung, omentum, pancreas, heart, kidney and brain). MMP 2 and MMP 9 also stained stromal fibroblasts. Staining was also observed normal epithelium and macrophages within metastatic foci.

### **MMPs and tumor stroma interaction**

Tumor environment is very important for expression and activities of MMPs. For instance IL12, a cytokine observed in the extracellular matrix, can enhance the activity of MMPs [38]. In the tumor cell-cell interactions, pericellular environment and products of degradation of the extracellular matrix are important for MMP production and activation [39]. Tumor cells also interact with stromal cells or cell-bound factors that stimulate the production of MMPs. Among these factors, extracellular matrix metalloproteinase inducer (EMMPRIN) stimulates in vitro production of MMPs. EMMPRIN is present at the surface of both tumor epithelial and peritumoral stromal cells. Stromal cells do not expressed EMMPRIN, but this molecule is bound to stromal cells via a superficial specific receptor. [36, 40] **(Figure 10).**

MMP3 and MMP2 are expressed predominately in peritumoral fibroblasts [5, 35]. MT-MMP1 is produced in fibroblasts and is a major activator of MMP2 this suggests that the stroma component is fundamental for MMP production. MT1-MMP is anchored to the cell surface and acts as a receptor for TIMP2 that binds to MT1-MMP through his N-terminal domain. This binary complex acts then as a receptor for pro-MMP2. TIMP2 C-terminal binds to pro-MMP2 and MT1-MMP cleaves then pro-MMP2 causing the formation of an intermediate species **(Figure 11).**

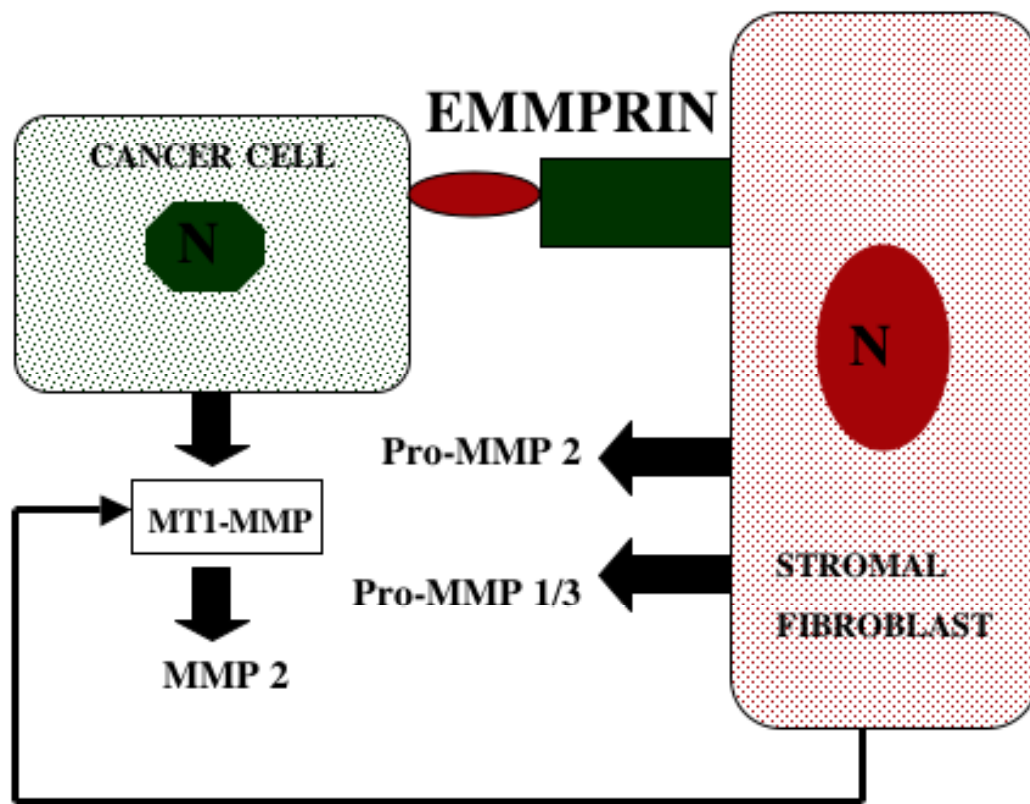


Figure 10. Effects of EMMPRIN in MMP secretion, activity and tumor cell and stroma cell interaction.

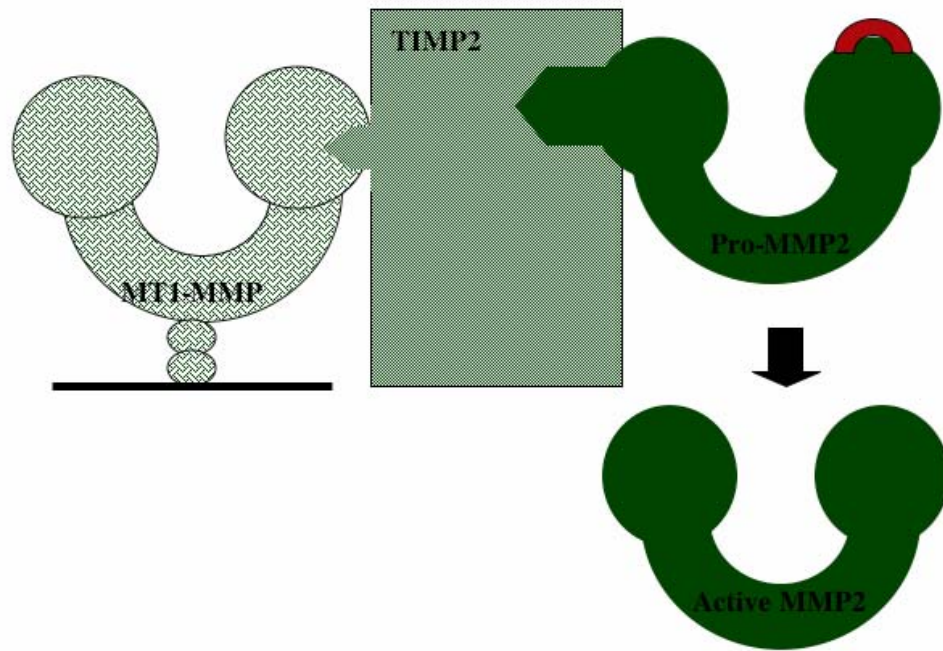


Figure 11. Role of MT1-MMP and TIMP2 in MMP2 activation.

Stromal fibroblasts at the tumor invasion front are thought to produce the bulk of MMP2. Tumor cells usually express low constitutive levels of MMP2. Stromal cells have strong but short induction of MMP2 [39]. This very high and complex regulation of the expression of MMPs represents a host response to the tumor and neoplastic cell interaction with the tumor stromal component is fundamental for cancer invasion and metastasis.

## Overview on tissue inhibitors of MMPs

The activities of MMPs are in part influenced by the presence of tissue inhibitors of metalloproteinases (TIMPs). An increase in the amount of TIMPs relative to MMPs could function to block tumor cell invasion and metastasis. In fact, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs. Inhibition of both MMPs and osteoclastic bone resorption could be efficacious treatment for prevention of osteolytic bone metastases [19]. Four homologous TIMPs have been characterized so far, TIMP1-4. They are low molecular weight proteins that bind to active MMPs in a 1:1 molar ratio, and form non-covalent tight complexes with them [40, 41].

**(I) TIMP1** can inhibit tumor growth, invasion, and metastasis in experimental models and it also exhibits growth factor-like activity and can inhibit angiogenesis [42]. Alternatively, down-regulations of TIMP1 have been reported to contribute significantly to the tumorigenic and invasive potentials. In addition to inhibiting tumor cell invasion and metastasis, overexpression of TIMPs in tumor cells also inhibits primary tumor growth [43]. Paradoxically, over-expression of TIMP1 appears to confer growth advantage on breast carcinoma cells in vivo. Node-positive patients showed significantly higher TIMP1 mRNA and antigen concentrations than those who were node negative, and patients co-expressing high levels of TIMP1 mRNA within the tumors had worse survival prognosis [44].

**(II) TIMP 2** has been recognized as an adaptor protein that activates pro-MMP-2. Enhanced TIMP2 expression may denote a stromal response to tumor invasion, indicative

of aggressive behavior in a subset of breast carcinomas. The role of TIMP2 in MMP2 activation is still under intense scrutiny. It is thought that the intermediate form of MMP2 forms a complex with MT-MMP1 and TIMP2. There is binding with MMP2 at the level of its homeopexin domain. This can be regarded as the first step of MMP2 activation. MT-MMP1 acts as a receptor for TIMP2, where the binary complex acts as a receptor for pro-MMP2. Cleavage at Asb37-Lau 38 occurs and an intermediate active species is formed.

Optimal TIMP2 levels are required for efficient pro-MMP2 activation [45]. There is also an independent way of activation of MMP2 that does not require TIMP2 [4]. Studies in cancer from human patients usually correlated the presence of TIMP2 with an increase in MMP expression and therefore worse prognosis [4, 46, 47, 48]. Multiple studies have been conducted of the role of TIMP2 in breast cancer. Experimental reports of *in vivo* conditions usually correlate overexpression of TIMP2 either by transfection, adenovirus delivery or retroviral delivery with reduced tumor growth and reduced metastatic behavior [4, 49, 50, 51].

**(III) TIMP 3** has been reported to be a possible tool for the analysis of cell cycle progression, terminal differentiation, and replicative senescence [52]. Overexpression of TIMP3 can inhibit angiogenesis and associated tumor growth [53]. Increased expression of TIMP3 resulted in a statistically significant suppression of breast cancer tumoral growth *in vitro*.

**(IV) TIMP 4** MMP inhibitory activity of the expressed TIMP4 protein has been reported. TIMP4 shares with other TIMPs the ability to block tumor cell invasion *in vitro* and, at least in some instances, it also blocks tumor growth and metastasis *in vivo* [41, 54,

55]. TIMP4 transfectants significantly inhibited tumor growth by 4-10-fold in primary tumor volumes, and in an axillary lymph node and breast cancer lung metastasis as compared with controls [56]. However recombinant TIMP4 also stimulated the growth of MDA-231 breast carcinoma cells. Administration of naked TIMP4 DNA significantly stimulated mammary tumorigenesis *in vivo* [43].

The exact function and mode of action of TIMPs is still yet to be determined as is the importance in preventing breast cancer metastasis *in vivo* and potential therapeutical use of these molecules.

### **MMPs in the central nervous system**

MMPs are expressed physiologically in central nervous system cells. The main MMPs produced in the CNS are MMP3, MMP2 and MMP9. Astrocytes secrete MMP2 and they also produce TIMP2. Peripheral growth cones produce and secrete MMPs. Other cells that also produce MMPs are the neurons, microglial cells and oligodendrocytes. Neurons express mainly MMP9 and MMP3. TIMP2 is the most abundant of tissue MMP inhibitor expressed in the neurons [57]. However in normal non-challenged brain the levels of MMPs produced are low. There are reports of numerous pathologic processes in the central nervous system that cause increase in expression and increase activity of MMPs. For instance, the degradation of the extracellular matrix in gliomas is thought to be in part due to the activity of MMPs, additionally MMPs regulate glioma vascularization and are correlated with glioma aggressiveness [58, 59, 60]. HIV nef protein disrupts the blood-



brain-barrier via MMP9 [61]. MMP9 is also responsible for the disruption of the blood brain barrier in the case of brain ischemic injury [62]. Ischemia activates MT1-MMP that increases MMP2 activity. MMP2 increases IL $\beta$ 1 causing up regulation of the NF- $\kappa$ B pathways that leads to an increase in MMP3 that activates MMP9 that increases the lesions on the blood brain barrier [63]. Disease mediated by HTLV1 (human T lymphoma virus) is also correlated with levels of MMP9 and MMP3 [64].

The mechanism of activation and regulation of MMPs in the central nervous system is not completely understood. Astrocytes have been correlated with MMP expression and activity. Co-culture with astrocytes is associated with increased invasion of glioma cells and production of MMP2 [65]. After reperfusion injury there is an increase expression of MMP2 in astrocytes [63]. Rat astrocytes stimulated with protein kinase C and LPS increase production of MMP9 and this is correlated with disease development [66, 67]. HTLV1 virus infects T lymphocytes that stimulate astrocyte production of MMP3 and MMP9 [64]. HIV virions are observed in astrocytes and cause increase of pro-MMP1 and pro-MMP2 [68]. Additionally, astrocytes may play an important role in the development of brain metastases of breast cancer. Breast cancer cells have been shown to respond to extracellular stimuli by producing many cytokines and growth factors that can modulate tumor cell proliferation, growth, and/or metastases. The growth-stimulatory effect was partially reversed by anti-IL-6, anti-transforming growth factor beta (anti-TGF beta), and anti-IGF-I antibodies, indicating that these metastatic cells use exogenous cytokines as paracrine growth factors. IL-6 produced a variety of responses in the different BC metastatic variants. Responses to exogenous IL-6 might determine the differences among these metastatic

variants by extending cell survival of selected subpopulations, giving them the opportunity to respond to growth factors or other favorable conditions. Cytokines produced by glial cells in vivo may contribute, in a paracrine manner, to the development of brain metastases by breast cancer cells [69, 70].

### **MMPs and MAPK**

The mitogen activated protein kinase (MAPK) pathway is one of most important transduction signaling pathways that is related to numerous pathogenic processes, including neoplasia. Genes that codify for molecules in this family, such as MKK4 and MAPKK4 are considered to be important in the metastatic cascade [71]. Moreover components of these pathways have been correlated with cancer invasion, and development. They have also been linked with MMP expression. Activation state of the ERK pathway in tumor cells correlates with the invasive phenotype, which was determined by the ability of cells to invade through reconstituted extracellular matrix. Elevated expression of MMP-3 was observed in tumor cells in which constitutive activation of the ERK pathway was detected. Blockade of the ERK pathway by treatment with PD184352, a specific and powerful inhibitor of MAPK/ERK kinase (MEK), suppressed the expression of MMP3 and inhibited markedly the invasiveness of tumor cells [72]. Up regulation of MMP3 mRNA by FGF-2 requires de novo protein synthesis and activation of the ERK-1/2 pathway. [73]. Inhibition of ERK's phosphorylation blocks the changes in MMP 3 and TIMP1 expression [74].

MMP2 mRNA expression, protein expression and gelatinolytic activity are correlated with ERK phosphorylative activity. MMP9 enzyme activity has an inverse relationship with phosphorylated ERK [72]. ERK/MAP kinase is essential for MMP9 up regulation via PKC and cytokine pathways in astrocytes [75]. Resident brain cells secrete MMP after mechanical injury, astrocytes are the main source of MMP9 activity, and ERK and p38 MAP kinases are unregulated after mechanical injury, and mediate the secretion of MMP-9. MEK inhibitor PD98059 inhibits MMP2 promoter activity and Sp1 phosphorylation. Overexpression of constitutively active MEK1 stimulates Sp1 phosphorylation and MMP2 promoter activity [76]

MMP11 expression correlates with decrease in breast cancer cell apoptosis and this is mediated through ERK1/2 [77]. The activation of MMP1 is related with p38 and/or ERK1/2 activity [78, 79]

### **Specific objectives**

Metalloproteinases are enzymatic proteins that have been extensively associated with breast cancer local invasion and metastatic process. Intensive studies in this area have tried to define the exact mechanism of action of MMPs, their importance in establishing breast cancer prognosis and the role of related proteins such as tissue inhibitors of MMPs (TIMPs). In view of the inherent difficulties in working with human cancerous tissue, the limited extrapolation of *in vitro* experimental data, as well as the paucity of studies of MMP expression in breast cancer brain metastasis, we propose the use of a rodent model

for distant metastasis of breast cancer to the brain to study MMP expression in metastatic breast cancer and to study TIMP-MMP interactions. **Our hypothesis is that MMP 2, 3 and 9 play a role in the development of brain and lung metastasis of breast cancer.**

**Objective 1:** Characterization expression of MMP2, 3 and 9 in brain and lung metastatic sites, compared with normal brain and lung tissues and breast cancer tumor cells growing in culture. To achieve this goal our analysis will focus on: (1) measure and quantify MMP2, -3 and -9 protein expression by western blot (WB) and immunohistochemistry (IHC);(2) quantify the expression of MMP 2,9 and 3 mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) and (3) evaluate activity of MMP 2 and 3 in metastatic foci by *in situ* (ISZ) and gel zymography.

**Objective 2:** Determination of roles of MMP 2, 3 and 9 in the development of BC brain and lung metastasis. (1) Determine if synthetic MMP inhibitor (PD-166793) will alter development of BC brain metastasis. (2) Determine if stable overexpression of TIMP 2 in BC cell lines will alter development of BC brain metastasis (**Figure 12**).

**Objective 3:** Investigate possible mechanisms of MMP2 activation in the brain. (1) *In vitro* study of the effect of astrocytes in MMP2 expression in a breast cancer cell line. (2) Evaluation of mitogen activated protein kinase (MAPK) components expression in cells stimulated by astrocytes factors. (3) Role of MAPK inhibitors in the expression of MMP2 by BC cells stimulated with astrocyte factors.

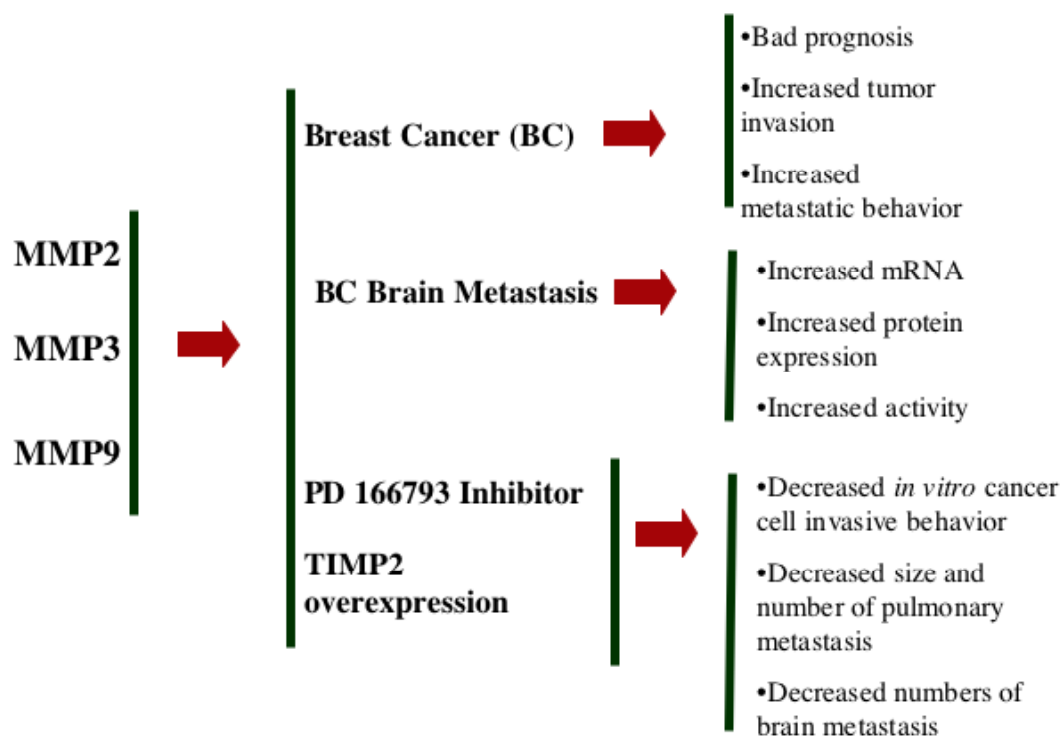


Figure 12. Effect of MMPs in metastasis development.

## Summary

In summary the objectives of this project are three fold. We pretend to characterize the expression of MMP2, - 3 and -9 in breast cancer brain and lung metastasis at the protein and mRNA level and evaluate the activity of these proteases in the tumor foci. Additionally, to evaluate if these molecules play a role in the development of brain and lung metastasis, inhibition of MMP2 activity will be attempted via TIMP2 overexpression and drug induced inactivation of MMPs by the use of PD 166793. Finally a series of *in vitro* experiments will be performed in order to evaluate the role of astrocytes and ERK1/2 in MMP regulation in breast cancer cells and breast cancer brain metastasis.

## **CHAPTER II**

### **EXPRESSION OF MMP2, MMP9 AND MMP3 IN BREAST CANCER BRAIN METASTASIS**

#### **Introduction**

The metastatic process of breast cancer (BC) has been the subject of intense scrutiny. The brain is one of the most common organs affected in the spread of BC that ultimately results in fatal overcome of the disease. Brain metastasis is an increasingly common complication in breast cancer patients. Approximately 15% to 30% of breast cancer patients develop brain metastasis [1, 2]. A suitable specific environment is important to the development of tumor cells [80]. The exact role of the brain environment to the development of the metastatic process has yet to be clarified. Many theories have been developed to study and understand metastatic behavior. Factors such as neoplastic cell molecular and genetic characteristics [4] and biological environment are thought to be determinants in the metastatic process.

Matrix Metalloproteinases (MMPs) are a broad family of zinc-dependent proteinases that play a key role in extracellular matrix degradation, implicated in numerous pathogenic processes [9]. Tumor cells are thought to secrete these matrix-degrading enzymes and/or induce host cells to elaborate them [39]. MMPs have been associated with pathology within the central nervous system in neoplastic disease, such as glioma and melanoma brain metastasis [81, 82], and non-neoplastic disease, such as trauma, ischemia

and immune-mediated disease [83]. MMPs have also been extensively studied in the context of breast cancer prognosis. Most studies to date have been performed in human tissue collected from patients diagnosed with breast cancer or in breast cancer cell lines. Most reports suggest that increased expression of MMP2, -3 and -9 proteins, correlates with worse prognosis [18, 6].

In this context, MMP2, -3 and -9 are thought to play an important role in breast cancer invasion, metastasis and tumor angiogenesis [84]. MMP2 over-expression and activation have been associated with the invasive potential of human tumors. Active MMP2 and MMP9 were detected more frequently in malignant than benign breast carcinomas; MMP3 was observed in highly invasive breast cancer cell lines [16]. Some reports, however, do not correlate MMP2 and MMP9 immunohistochemical staining with the presence of metastases at the time of diagnosis or with disease outcome [17]; absence of distinct positive immunoreactivity for MMP2, -3 and -9 has been observed in both invasive and non-invasive tumor cells without apparent differences in the staining intensity [18]. In this regard, additional *in vivo* studies that characterize MMP expression in metastasis are needed.

Few studies are available on the expression of MMPs within breast cancer metastasis [38, 7] and, to our knowledge no one has characterized the expression and activity of these molecules in breast-to-brain cancer metastasis. It is important to determine if MMPs have different effects/roles in the development of metastasis in different organs because this may help to understand why BC cells metastasize to preferential organs. Here we focused on the metastatic process of BC to the brain in a rodent model. This

understanding could be utilized in the development of the current therapeutic approach to metastatic cancer.

The metastatic rodent models for breast cancer described to date study mostly nodal, pulmonary and bone metastasis [10]. In most, brain metastases occur as a non-predictable event and only sporadically. The use of animal models to study *in vivo* tumor progression and metastatic behavior is important to understand the mechanism of metastasis development. It is also an important tool for pharmacological evaluation of cancer therapy. Several synthetic MMP inhibitors are under investigation for clinical trials in patients with cancer as they are thought to inhibit both primary tumor invasion and metastasis. [7].

We here used an *in vivo* model that consistently produces brain metastasis [80] to evaluate the expression and activity of MMP2, -3 and -9 in metastatic foci of BC in the brain. We found the levels of MMP2, -3 and -9 mRNA and protein, in BC brain metastasis are higher than those of normal brain tissue. Additionally, the activities of MMP2 and MMP3 in metastatic foci are higher than in non-affected brains. We also demonstrated that MMP inhibition, by a specific MMP inhibitor decreases *in vitro* and *in vivo* cell invasive and metastatic behavior. To our knowledge, this is the first report of characterization of these molecules in brain metastasis of breast cancer in a rat model.



## Materials and methods

### *Tumor cell line*

The ENU1564 tumor cell line used in this study was developed in our laboratory and originated from an N-ethyl-N nitrosourea-induced mammary adenocarcinoma in a female Berlin-Druckrey IV (BD-IV) rat. This cell line is highly metastatic to brain and bone tissues [80].

### *Rat inoculation*

Forty-day-old BD-IV rats were used. The animals were obtained from a colony maintained at Texas A& M University in accordance with institutional animal care guidelines. The syngeneic animals were inoculated with  $1 \times 10^4$  tumor cells in the left ventricle. Inoculation was performed on animals under Ketamine (87mg/kg, intramuscular injection) anesthesia. The animals were humanely euthanatized using Pentobarbital (150mg/kg, intraperitoneal injection) when showing clinical signs of discomfort such as decrease response to stimulus. Complete necropsies were performed and tissues were sampled for histologic evaluation.

### *Tumor collection*

Brain samples were collected immediately after animal's death and placed on powdered dry ice until completely frozen. Samples were then kept at  $-80^{\circ}\text{C}$ . The samples

from metastatic tissue were collected from frozen brain sections. The half of the brain that was frozen in powdered dry ice was sectioned using a cryostat in 12 $\mu$ m sections and placed on gelatin-covered slides. Every fifth slide was stained with thionin stain prepared from a stock 1.3% thionin (wt/vol in H<sub>2</sub>O). Metastatic foci were identified under light microscopy. This information was then used to dissect the metastatic tumor, from frozen brain tissue sections. Immediately after dissection another 12 $\mu$ m section was stained in order to confirm the accuracy of the dissection.

#### *Immunohistochemistry (IHC)*

Five-micron (5  $\mu$ m) paraffin-embedded sections and 12 $\mu$ m frozen sections were used. Deparaffinization, rehydration and antigen-retrieval were done by immersion of slides in DECLERE<sup>®</sup> (Cell Marque, Hot Springs, AR) commercially available buffer in moist heat (pressure cooker) for 15 minutes. Potential non-specific binding sites were blocked with 5% normal goat or rabbit serum in PBS. After blocking, the sections were incubated with primary antibodies purchased from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA), in dilutions of 1:200 for MMP2, and 1:25 for MMP3 and -9. After three five-minute washes in PBS, the sections were then incubated with either biotin-conjugated anti-rabbit or anti-goat IgG (Vector Laboratories, Burlingame, CA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development. Counterstaining was done with diluted hematoxylin. Sections that were not incubated with primary antibody served as negative control.

### *Western blotting (WB)*

After microscopic dissection of frozen brain specimens, the tissue was homogenized in lysis buffer at a 1V: 10V dilution (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0, and 0.1% SDS), supplemented with a mixture of protease inhibitors. Samples were run on a 9-12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated one hour in blocking buffer (20 mM Tris-HCl buffered saline containing 5% nonfat milk powder and 0.1% Tween 20). Blots were incubated at 4°C overnight with anti-MMP2 (1:2000); MMP3 (1:500) and MMP9 (1:1000) (all antibodies from Santa Cruz Biotech, Santa Cruz, CA), washed extensively and then incubated for one hour with a 1:5000 dilution of secondary anti-rabbit or anti-goat antibody. After additional washes, the blots were incubated with chemiluminescent substrate, according to the directions in the kit (SuperSignal<sup>®</sup> West Pico, Pierce, Rockford, IL).

### *Reverse transcription-PCR*

We extracted total RNA from frozen specimens using Trizol reagent (Invitrogen, Gaithersburg, MD). First-strand cDNA was primed with oligo (dT), synthesized using RETROscript kit (Ambion, Austin, TX), and served as a template reverse transcription-PCR (RT-PCR). PCR primers were as follows:

- MMP2 primers (forward, GACCTGACCAGAACACCATCG; reverse, 5'-GCTGTATTCCCGACCGTTGAAC-3');

- MMP3 primers (forward, 5'-CCTCTATGGACCTCCCACAGAATC-3'; reverse 5'-GTGCCAATGCCTGGAAAGTTC-3');
- MMP9 primers (forward, 5'- CCCCATTACTTTGGAAACGC-3'; reverse, 5'-ACCCACGACGATACAGATGCTG-3');

Rat MMP2, -3 and-9 cDNA sequences were obtained from the <http://www.ncbi.nlm.nih.gov> web site and MacVector<sup>®</sup> (version 7.0, Accelrys, San Diego, CA) software was used to design the primers. To demonstrate the integrity of the RNA samples used in the RT-PCR reactions, parallel amplifications with oligonucleotide primers for mouse  $\beta$ -actin cDNA (forward, 5'- ATGTACGTAAGCCAGGC-3'; reverse, 5'-AAGGAACTGGAAAAGAGC -3') were performed.

#### *Fluorescent-labeled substrate-based in situ zymography*

Zymography is the choice method for evaluation of MMP2 and -9 activities [85]. Because of the small size of the metastatic foci and the paucity of material collected, we opted for an *in situ* method [86]. The substrate was prepared by dissolving 0.1% fluorescent-labeled substrate (Molecular probes, Eugene, OR) in gelatin according to manufacturer's instructions. 50  $\mu$ l of substrate gel solution were pipetted onto the slide and evenly distributed on the slide surface. Frozen sections (10-15 $\mu$ m) were placed on the coated slide. The slides were incubated in a moist box with Tris-buffer (pH 7.4) and the box was wrapped in foil to protect from light and placed at 37°C. Results of *in situ*

zymography were evaluated after 48 hours of incubation. The samples were examined microscopically under UV light. Control samples were stained with thionin stain.

### *Gel zymography*

Gelatin zymography was performed as described previously [7]. In brief, samples were electrophoresed on 10% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) gelatin and Ready Gel Zymogram Gel 12%, casein (Biorad, Hercules, CA). After electrophoresis, the gels were washed twice for 30 min each in 2.5% (vol/vol) Triton X-100 at room temperature and then incubated in substrate reaction buffer (50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, 0.02% [wt/vol] NaN<sub>3</sub>, pH 8.0) for 8 to 18 h at 37°C with gentle shaking. The gels were then stained with Coomassie Blue R250 in 10% (vol/vol) acetic acid and 30% (vol/vol) methanol for 1 to 2 h and destained briefly in the same solution without dye. Proteolytic activities were detected by clear bands indicating the lysis of the substrate. Quantification of band density was carried out using Flour S MultiImager<sup>®</sup> technology from Biorad (Hercules, CA).

*In vitro and in vivo MMP inhibition assay*

PD 166793 (S-2-4'-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) was kindly provided by Dr J T Peterson (Cancer Molecular Sciences, Pfizer Global Research & Development, Ann Arbor, MI).

*In vitro* invasiveness was evaluated using the method previously described [29]. Boyden chambers were used for the invasion assay. Briefly, each Boyden chamber (Becton Dickinson Biosciences, USA) consists of a BD Falcon TC Companion Plate with Falcon Cell Culture inserts containing an 8µm pore PET membrane with a thin layer of Matrigel basement membrane matrix. First, the interior of the inserts was rehydrated for 2h with warm (37 °C) bicarbonate based culture medium. The upper chambers were filled with 0.5 ml of cell suspension ( $1.25 \times 10^5$  cells/ml) in Dulbecco's Modified Eagle Medium (Invitrogen Corporation, Carlsbad, California). The same media including 10% fetal bovine, was placed in the lower chambers as chemoattractant. Experiments using 10µM PD166793 MMP inhibitor, in both chambers media, were conducted in parallel [87]. The chambers were incubated at 37 °C in a humidified atmosphere of 5% carbon dioxide for 24 h, non-migrating ENU1564 cells on the upper surface of the inserts were removed by wiping with a cotton swab, and the migrating cells on the lower surface were fixed and stained with Insta Stain 3 Step (S&K Reagent, Inc, Denver, CO). The invasive potential was quantified by counting the total number of cells on the lower surface of the inserts under a light microscope at 400X magnification. Three random visual fields were counted for each assay. Each invasion experiment was carried out in triplicate.

To evaluate *in vivo* inhibition of MMP evaluation we conducted the following experiment. Ten BD-IV rats were inoculated with  $1 \times 10^4$  ENU1564 via left ventricle. Five animals were selected randomly for the control group and for the drug treatment group. Animals from the control group were daily inoculated via peritoneum with vehicle only (DMSO). The five animals in the treatment group were treated daily with intraperitoneal injection of 5mg/kg of PD 166793 as described previously [87]. The study had the duration of 24 days. All animals were sacrificed at the end of the study. Six sections of brain per animal were evaluated histologically, and foci of breast cancer brain metastasis were counted.

#### *Statistical analysis*

Paired T-student tests were performed with densitometry values obtained from Western blotting-autoradiographs analysis and by photograph zymography results using Flour S MultiImager<sup>®</sup> technology from Biorad (Hercules, CA). Differences were considered statistically significant when P was  $\leq 0.05$ .

## **Results**

#### *Histological evaluation of brain metastatic foci*

Six weeks after inoculation the animals began to show neurological signs, such as depression and/or head tilt, and were humanely euthanatized. No macroscopic

abnormalities were observed in the central nervous system upon necropsy evaluation. Histological evaluation of the brain revealed intra-cerebral metastatic neoplasia. Morphologically, small clusters of epithelial neoplastic cells resembling the cultured cell line could be observed. The neoplastic foci were scattered randomly throughout the brain, affecting more frequently the caudal aspect of the parietal lobes and cerebellum (**Figure 13**).

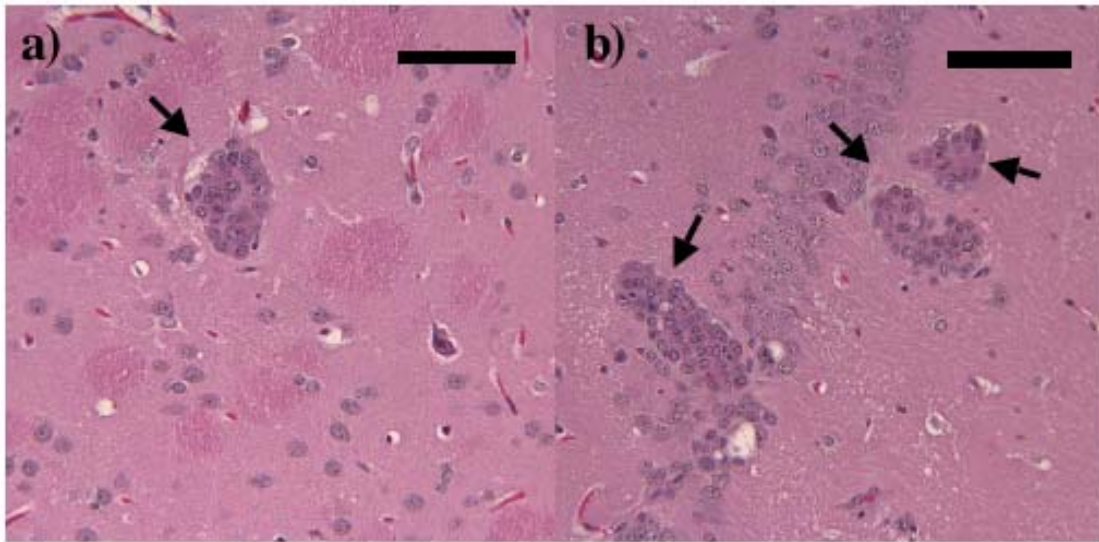


Figure 13. Histology of metastasis of breast cancer to the brain. The metastatic foci in the brain are observed as small clusters of epithelial neoplastic cells scattered randomly throughout the brain. Arrows indicate metastatic foci. Bar indicates 100 $\mu$ m.

The neoplastic foci varied in size from five to two hundred-micron. The estimated number of tumor foci per animal brain varied from five to fifty (**Table 2**).



**Table 2.** Morphological characterization of brain metastatic foci.

	<b>Tumor diameter (<math>\mu\text{m}</math>)</b>	<b>Number of tumor foci per brain section</b>
<b>Brain metastasis</b>	43.3 $\pm$ 34.92	24 $\pm$ 15.44

Values are mean  $\pm$  standard deviation.

The tumor foci had absence of fibrous stroma, inflammatory cells or necrosis. Mild to marked astrocyte reactivity was observed around the neoplastic cells. Metastatic tumors in other organs, such as bone, lung and pancreas, were also observed (**Table 3**).

**Table 3.** Organ distribution of breast cancer metastatic foci.

<b>Metastatic sites</b>	<b>Brain</b>	<b>Lung</b>	<b>Bone</b>	<b>Kidney</b>	<b>Panc reas</b>
<b>Numbers of animals affected/ total number of animals examined</b>	6/10	10/10	4/5	3/5	1/5

#### *Immunohistochemistry for MMP2, -3 and -9 proteins in brain metastatic foci*

Immunohistochemistry was performed in order to characterize MMP-protein expression within the metastatic brain foci. Immunohistochemical staining for MMP2 showed immunolabeling with moderate intensity in the cytoplasm of neoplastic cells within the brain metastatic foci (**Figure 14(a) and (b)**). In addition astrocytes, microglial cells and

endothelial cells also had mild staining. MMP3 staining was observed with strong intensity in the cytoplasm of neoplastic cells (**Figure 14(g) and (h)**). Mild staining was also observed in astrocytes. MMP9 staining was weak in the neoplastic cell cytoplasm and faint staining of glial cells was also observed (**Figure 14(d) and (e)**). Similar results were observed in neoplastic epithelial cells in the lung metastatic foci (data not shown).

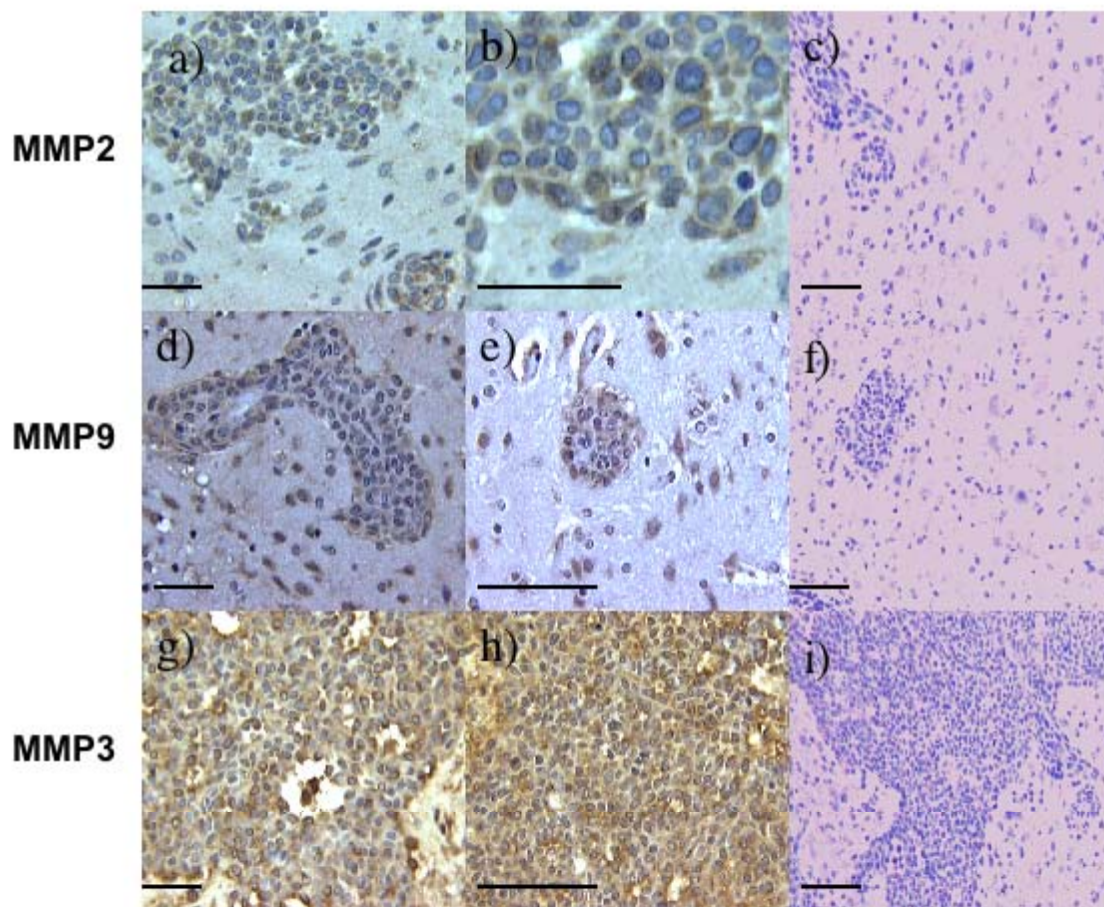


Figure 14. Localization of MMP2, -3 and -9 in the brain metastatic foci. Immunohistochemical staining (brown) of MMP2 (a&b), MMP3(g&h) and MMP 9(d&e) protein in the brain metastatic foci revealed positivity within neoplastic cell cytoplasm. Negative controls for MMP2, -3 and -9 are respectively c, i and f. Note that glial cells are also positive. Bars indicate 100 $\mu$ m.

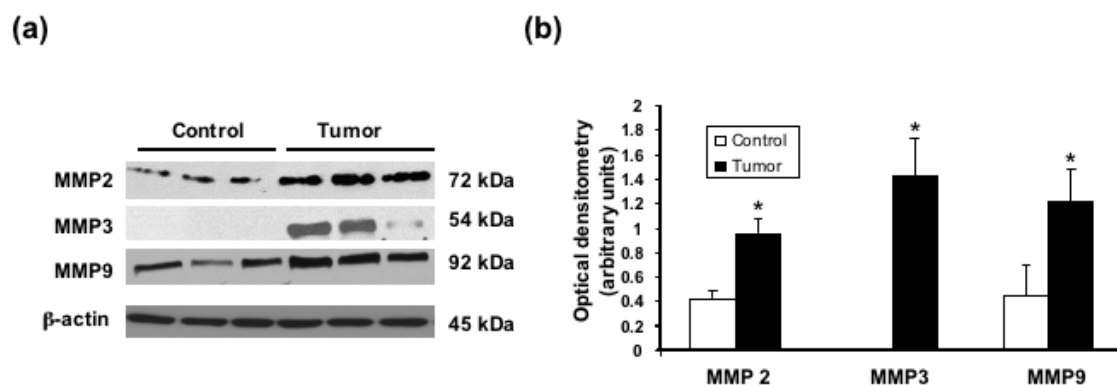


Figure 15. Increased expression of MMP2, -3 and -9 protein in the metastatic brain foci.

(a) Evaluation of protein expression by Western blotting. The membranes were stripped and re-probed with  $\beta$ -actin antibody to confirm equal loading. (b) Quantitative analysis of MMP2, -3 and -9 expression was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (\*) for statistically significant when P was  $\leq$  0.05).

*Increased expression of MMP2, -3 and -9 in brain metastatic foci*

To confirm IHC results on MMP-2, -3 and -9 protein expression and in order to semi-quantify protein expression, we extracted protein from the metastatic neoplastic tissue. Evaluation of protein expression by Western blotting revealed that MMP3 expression was significantly higher in neoplastic brain metastasis tissue when compared with control tissue from brains of age-matched-non-inoculated rats ( $p \leq 0.032$ ) (**Figure 15**).

Such difference was not observed in lung metastatic foci (data not shown). MMP2 was also more significantly expressed in brain metastasis of breast cancer when compared with normal brain control tissue ( $p \leq 0.014$ ). MMP 9 expression was also significantly higher in tumor tissue ( $p \leq 0.049$ ).

To confirm IHC and WB results on MMP-2, -3 and -9 protein expression, we extracted total RNA from frozen specimens. Semi-quantitative RT-PCR analysis of MMP-2, -3, and -9 mRNA of brain metastatic foci of breast cancer was compared with mRNA obtained from age-matched non-inoculated rats. The comparison revealed that the amounts of MMP-2, -3, and -9 mRNAs of brain metastasis foci of breast cancer were higher than those of control tissues. This data is compatible with the WB results. MMP3 mRNA was more abundant in neoplastic tissue when compared with lower expression in controls. The same was observed with MMP2 and MMP9 (**Figure 16**).

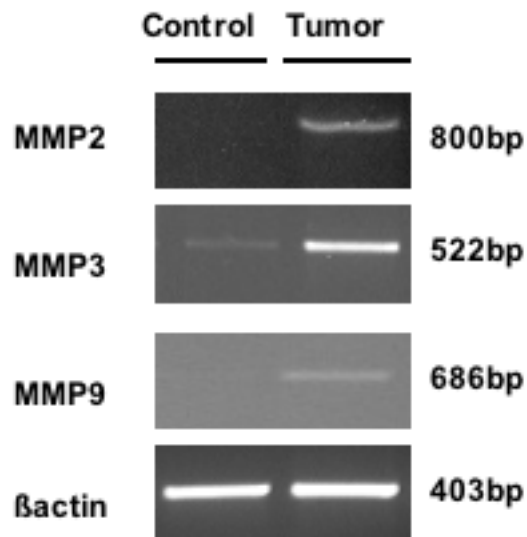


Figure 16. Increased expression of MMP2, -3 and -9 mRNA in the metastatic brain foci. Semi-quantitative RT-PCR analysis was used to detect MMP2, -3, -9 and  $\beta$ -actin in total RNAs from normal brain and metastatic brain foci.  $\beta$ -actin was used as an internal control.

*Increased MMP2 and MMP3 activity in brain metastatic foci*

To determine if the higher expression of MMPs was correlated with intra-tumoral increased enzyme activity, we performed zymography studies. *In situ* zymography revealed intratumoral gelatinase (MMP2 and/or MMP9) activity characterized by loss of fluorescence (**Figure 17**). Additionally gel zymography showed that there was a significant increase in both MMP 3 and MMP2 activities ( $p < 0.05$ ) (**Figure 18**). MMP9 activity was not detected on the samples evaluated.

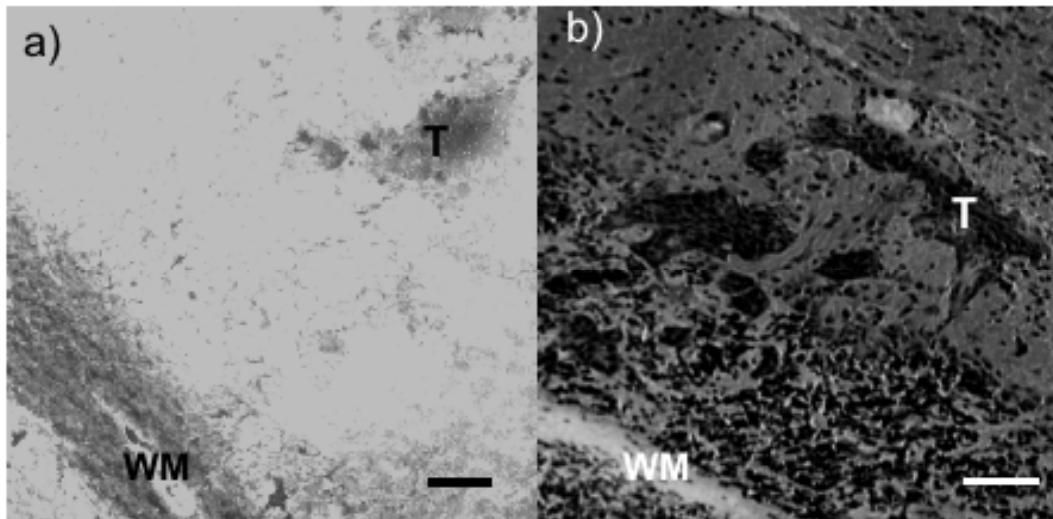


Figure 17. Evaluation of gelatinase activity in the brain foci by *in situ* zymography. (a) Frozen section observed under UV microscope. Marked gelatinase activity (loss of fluorescence) was observed within the tumor foci. (b) Thionin stain of the same area. WM represents white matter; T indicates tumor foci. Bars indicate 100µm.

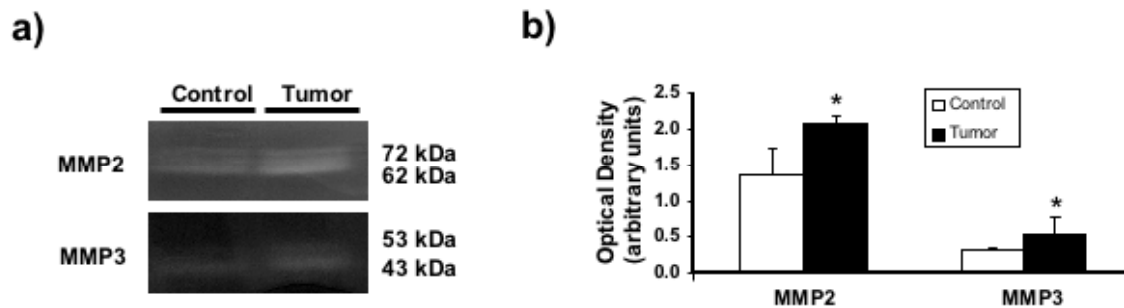


Figure 18. Increased MMP2 and MMP3 enzymatic activities in the metastatic brain foci. (a) Evaluation of MMP2 and MMP3 activities by gel zymography. (b) Quantitative analysis of MMP2, and MMP3 activity was determined by densitometry of respective active bands (62kDa and 45kDa). The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (\*) for statistically significant when  $p \leq 0.05$ .

#### *Effect of MMP inhibitor, PD 166793 on the in vitro invasive potential of ENU1564 cells*

To determine whether the use of an MMP inhibitor (PD166739) has any influence on ENU1564 cells *in vitro* invasive potential a Matrigel-based invasion assay was performed. Boyden chamber chemoinvasion analysis showed that PD 166739 significantly reduced ( $p \leq 0.001$ ) the number of ENU1564 cells that invaded the Matrigel when compared with non-treated control cells (**Figure 19**).

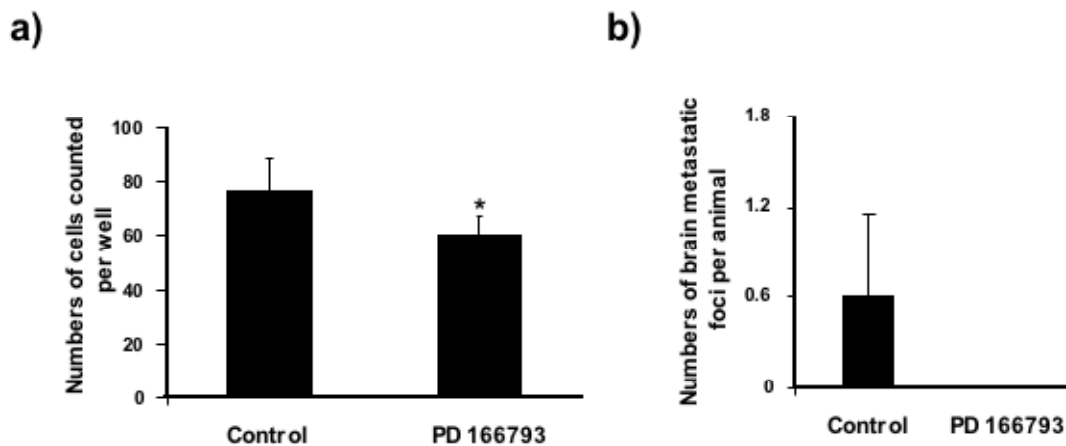


Figure 19. Decreased metastatic potential of ENU 1564 by PD 166793, a selective MMP inhibitor. (a) *In vitro* invasion chamber assay for ENU1564 cells was performed as described in material and methods. The results shown in the histogram are the mean  $\pm$  standard deviation of two individual experiments run in triplicate. (b) Numbers of metastatic foci in the brain in control ( $n=5$ ) versus animals ( $n=5$ ) treated with PD 166793. (\*) for statistically significant when  $p \leq 0.05$ .

#### *Effect of MMP inhibitor, PD166793 on brain metastasis of ENU1564 cells*

To determine if MMPs play a role on breast cancer brain metastasis we inhibited MMPs' activities by treating animals with PD166793, a selective MMP inhibitor. The animals in the control group started to show neurological signs such as depression and obnubilation at day 24 post inoculation, and all animals were sacrificed at that time. Gross evaluation did not reveal any significant changes in the central nervous system. Upon histological evaluation three of the five animals in the control group had presence of brain



metastasis. Brain metastases were not detected in the brains of animals treated with PD 166793 ( $p \leq 0.03$ ) (**Figure 19**).

## Discussion

In the present study, we found increased expression of protein and mRNA of MMP2, -3 and -9 in BC brain metastasis, suggesting that these molecules may be relevant in the metastatic process of breast cancer to the brain in our rat model. We also determined that there is a correlation between MMP expression and enzymatic activity within the neoplastic foci, and that inhibition of MMPs' activities reduces the metastatic potential of breast cancer cells *in vitro* and *in vivo*.

We used brains of age-matched BD-IV rats as controls, assuming that differences in MMP expression would be attributable to the presence of tumors. Although, ENU1564 expresses low levels of MMP2 and no detectable MMP3 or MMP9 *in vitro* (data not shown), our results show that MMP2, -3 and -9 protein levels in metastatic foci had significantly higher expression than controls. This difference was especially marked for MMP3 with undetectable protein expression in the control tissue. Because brain cells are positive for MMP3 by IHC it is likely that the levels of MMP3 protein, although present, are too low to detect by the WB procedure performed. To ascertain whether increased expression of MMP2, -3 and -9 proteins is correlated with their mRNA expression, cDNA was prepared from dissected tumor samples and RT-PCR was performed. Although this

technique is semi-quantitative our results show that there are higher levels of MMP2, -3 and -9 mRNA in metastatic neoplastic foci than in normal brain tissue.

*In situ* zymography results showed that there is a multifocal sharp increase in gelatinase activity and that it is morphologically associated with the neoplastic foci. Gel zymography results confirm the increase in gelatinase activity of MMP2 in tumor brain foci; however in spite of multiple technical variations we were unable to detect MMP9 activity in the samples evaluated. This result suggests that either the active levels of MMP9 in brain samples are under the detection limits of our technique or that MMP9 may play a different role from active MMP2 in the early development of brain metastasis since we were able to detect MMP9 activity under the same conditions in lung and mammary gland samples (data not shown). Additionally we observed a significant increase in MMP3 activity in the brain metastatic samples. Altogether these results suggest that MMP2 and MMP3 play a role in the metastatic process.

Our results show that the levels of MMP2 and MMP3 protein and mRNA are increased in neoplastic foci, which corresponds to an increase in intratumoral enzymatic activity. These results are in accordance with previous reports correlating MMP activity with metastatic and invasive behavior [9]. Previous studies that describe MMP expression correlated with breast cancer metastasis reveal that MMPs may be important in the metastatic process. MMP2 is related to osteoclastic resorption in the metastatic process to the bone [20]. MMP2 and MMP9 latent forms are released in breast cancer cells in co-cultures with bone extracellular matrix [19]. Additionally, incidence of metastasis to the brain was increased in animals injected intra-cardiac with clones of breast cancer cells

transfected with MMP2 [10]. Transfection with TIMPs has been reported to decrease cancer metastatic behavior [33, 34]. Conversely, TIMP evaluation in breast cancer patients has been associated with better prognosis [35]. This data correlates with the fact that MMPs have been associated with invasive and metastatic behavior of BC. High MMP2 serum levels are associated with adverse prognosis in node-positive BC, implying that this molecule is related to nodal metastasis [9]. Additionally, MMP2 and MMP9 protein expression have been extensively correlated with poor breast cancer prognosis, and survival rates that are invariably associated with metastatic and invasive BC phenotypes [37, 84].

The determination of what cell component of the tumor mass expresses MMPs is important in order to understand the role of these molecules in tumor development. Some studies have localized MMP2 to neoplastic epithelial cells. Others, however, associate them with different components of the tumor stroma and/or angiogenic blood vessels [88]. MMP9 has been associated with neoplastic cell plasma membrane ; non-neoplastic ducts and acini; stromal fibroblasts; endothelial cells; and tumor-infiltrating inflammatory cells, including neutrophils, macrophages, and lymphocytes. Expression of MMP3 was observed in both tumor and stroma cells [38]. Normal breast epithelia was weakly positive for MMP3 mRNA [17].

In concurrence with previous reports [18], we observed MMP2 expression in epithelial cancer cells. However, the IHC staining was observed diffusely within the cytoplasm instead of the neoplastic cell plasma membrane [17]. Due to the absence of stroma in our tumor we were unable to determine if stromal cells or angiogenic blood vessels were positive. Moreover, as described in previous reports [7, 10], MMP3 and

MMP9 staining was observed diffusely in the neoplastic cell cytoplasm with weaker staining for MMP9. Again, stroma evaluation was not possible. The variation in the intensity of staining may be due to different roles of these molecules in the metastatic process. However, IHC is not a good method to quantify expression and conclusions should be drawn very cautiously.

Stromal fibroblasts are thought to be important in stimulating the production of MMPs [39, 40, 41, 42]. In our model, the extreme paucity of fibrous stroma, the absence of necrosis and inflammatory cell infiltrate (such as macrophage invasion) within the brain metastasis are highly suggestive of an alternate mode of MMP activation in this particular type of neoplasia within the central nervous system, and questions the need and role of inflammatory/macrophage infiltrate and fibroblast presence in the expression and activity of MMP molecules. We characterized morphologically the brain metastatic neoplasia together with MMP expression and activity, and concluded that it may be independent of the presence of inflammation and fibrous stroma-interaction.

More must be known in order to fully understand the mechanisms that regulate MMP activity within the central nervous system. MMP2, -3 and -9 are expressed in normal brain tissue [63], MMP2, -3 and -9 are produced by neurons, astrocytes, glial cells and oligodendrocytes. MMPs have also been associated with intra-cerebral tumor evolution, MMP2 with glioma *in situ* invasion, and MMP9 with intratumoral angiogenesis. Astrocytes are thought to play a role in MMP9 activation and expression [66]. Astrocytic factors, such as TNF $\alpha$ , IL6 receptor, have been identified in cell cultures derived from metastasis of BC in the brain [4, 47]. Because of the growing evidence that astrocytes and/or glial cells have

a role in the MMP cascade in the central nervous system, we analyzed how these cells react and express MMPs in our experimental conditions. Using Hematoxylin & Eosin histological stain we observed that there is marked astrocytic reactivity around neoplastic foci. Peritumoral astrocytic reactivity was confirmed using GFAP (glial fibrillary astrocytic protein) immunohistochemistry staining (data not shown). MMP2, -3 and -9 staining of astrocytes and microglial cells was observed. The marked positivity of glial cells and astrocytes for MMPs, together with reactivity of these around the tumor, suggest that these may indeed play a role within the MMP cascade [48]. Nonetheless, the presence of glial staining suggests that further studies are needed in order to characterize the role of those cells in MMP cascade in metastatic disease to the brain.

MMP inhibitors are being investigated as an important tool for cancer treatment. [7]. In order to determine if MMPs play a role on breast cancer brain metastasis development we used PD 166739 (S-2-4'-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) as a selective MMP inhibitor that is known to decrease activities of MMP2, -3 and -9 [49]. Unlike the first generation of MMP inhibitors, PD 166793 does not inhibit other metalloproteinases such as TNF-alpha-convertase [30]. We observed slight but significant decrease in *in vitro* ENU 1564 invasion behavior when cells are in presence of PD 166793. Surprisingly, we observed a dramatic decrease in development of brain metastasis in animals treated with PD 166739. The disparity observed *in vivo* vs *in vitro* results may be associated with low levels of *in vitro* MMP expression; however these *in vivo* results are in concordance with our other *in vivo* results and strongly suggest that MMPs are important in the brain metastatic process of breast cancer.

In conclusion, we were able to use, for the first time, a rat model for distant breast cancer metastasis to the brain to successfully study expression and activity of MMP 2, -3 and -9. Our results indicate that MMPs are involved in breast cancer metastasis to the brain in our model.

## **CHAPTER III**

### **ROLE OF MMP2, MMP3 AND MMP9 IN THE DEVELOPMENT OF BREAST CANCER BRAIN METASTASIS IN A RAT MODEL**

#### **Introduction**

Brain metastases of breast cancer (BC) occur in 15-30% of BC patients and they usually are a late event in the disease process [70]. Few studies characterized BC-brain metastases, and evaluated the role of MMPs in this disease. We previously reported that brain metastases of breast cancer have high expression and activity of MMP2.

MMP2 belongs to a broad family of zinc-dependent proteinases that are important in extracellular matrix degradation. It is implicated in numerous pathogenic processes, such as cancer metastases [9]. Tumor cells secrete this matrix-degrading enzyme and/or induce host cells to elaborate it [38].

TIMP2 is reported to be the physiologic inhibitor of MMP2. An increase in the amount of TIMP2 relative to MMP2 may decrease MMP2 activity and block tumor cell invasion and metastases. However, the role of TIMP2 in cancer development is still under investigation. Although TIMP2 was at first considered a suppressor of invasion and metastases, the complexity of TIMP2/MMP2 interactions led to reconsideration of the role of TIMP2 in cancer [4]. TIMP2 expression in BC patients has been correlated with advanced disease, decrease of survival time, increase in tumor size, node positive status and tumor recurrence [46, 48, 50]. Paradoxically, genetic manipulation of cancer cells has

correlated experimental TIMP2 overexpression with decreased metastatic behavior. Here we propose to determine whether MMP2 is important to the development of brain metastases of BC in a syngeneic animal model, by over expressing TIMP2 in ENU 1564 cells.

MMPs have been extensively correlated with pathological processes within the central nervous system (CNS) [66]. They have been involved in the degradation of extracellular periphery of brain tumors such as glioma [58], as well as in glioma invasion [60] and vascularization [61]. Although the exact mechanism of activation and function of MMPs in brain disease is still under intense scrutiny, it is reported that astrocyte co-culture with glioma cells increases activation of MMP2 [20]. In addition, astrocytes can produce and/or regulate MMP2 production [57, 89]. Astrocytes also have been implicated in the mechanism of activation of other MMPs in the CNS [67]. Additionally, astrocytes may be involved in the development of BC brain metastases [69]. Astrocyte factors such as interleukin 6 (IL6), fibroblast growth factor-b (FGFb), and insulin-like growth factor (IGF) receptor are up regulated in breast cancer brain metastases.

Mitogen activated protein kinase (MAPK) pathway components, such as ERK1/2, have been related to MMPs activation and expression, have been associated with astrocytic activity and are considered genes related with tumor metastases [90].

We aim here to study the role of MMP2 in the development of brain metastasis in a rat syngeneic model, and to investigate ERK1/2 signaling pathway in astrocyte-mediated MMP2 response. We report results that suggest that MMP2 may play a role in the development of breast cancer brain metastases. Furthermore, we present evidence that



astrocytes are important for MMP2 up regulation in BC brain metastasis through activation of the ERK1/2 signaling pathway.

## **Materials and methods**

### *Cell lines and cell culture*

The ENU1564 tumor cell line used in this study was developed in our laboratory and originated from an N-ethyl-N nitrosourea-induced mammary adenocarcinoma in a female Berlin-Druckrey IV (BD-IV) rat. This cell line is highly metastatic to brain and bone tissues [27]. The primary rat astrocyte CTX-TNA2 cell line used was purchased from ATCC (VA, USA). Both cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) DMEM supplemented with 10% fetal bovine serum (Invitrogen) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). All cells were grown at 37°C in a humidified incubator containing 5% CO<sub>2</sub> in air. Cells were passaged biweekly and used for experiments when in the exponential growth phase. Rat astrocyte conditioned media was obtained as previously described [28, 29]. Briefly, the cells were cultured for 9 days and replenished with medium containing 10% FBS on days 0, 3, and 6. On day 8, the 48h conditioned medium was collected, centrifuged, and filtered.

### *Transfection experiment*

Dr Stetler-Stevenson, (NIH, USA) graciously provided the TIMP2 human plasmid [30]. TIMP2 human sequence has high homology (more than 92%) with the respective TIMP2 rat sequence (92%, BLAST). pcDNA-TIMP2 was generated by subcloning TIMP2 cDNA into the pcDNA3.1 vector (Invitrogen). TIMP2 cDNA was released by cutting with Pst1 and Apa1 restriction enzymes, gel purified, blunt-ended ligated into blunt-ended Xba1 site of pcDNA 3.1. The orientation of cDNA was verified by restriction enzyme digestion and sequencing. The vector has a eukaryotic selection marker (neomycin resistance gene) that allows selection under G418 (Invitrogen). After linearization with Nru1, the plasmid was transfected into ENU1564 cells using Lipofectamine2000 (Invitrogen). After transfection, the cells were placed in antibiotic selective media (G418) to select clones that had successfully been transfected with the plasmid. The colonies were screened by WB for the expression of TIMP2 protein. Animals were inoculated with the stably transfected ENU1564-TIMP2 cell line. The animal inoculation was done in groups of nine animals. Nine control animals were inoculated with ENU1564 cells at the same time.

### *Invasion assay*

*In vitro* invasiveness was evaluated using the method previously described [31]. Boyden invasion chambers were used. Briefly, each Boyden chamber (Becton Dickinson Biosciences, NJ, USA) consists of a BD Falcon TC Companion Plate with Falcon Cell Culture inserts containing an 8- $\mu$ m pore PET membrane with a thin layer of Matrigel

basement membrane matrix. First, the interior of the inserts was rehydrated for 2h with warm (37°C) bicarbonate based culture medium. The upper chambers were filled with 0.5 ml of cell suspension ( $1.25 \times 10^5$  cells/ml) of ENU1564 and ENU1564-TIMP2 cells. Ten percent fetal bovine serum in Dulbecco's Modified Eagle Medium (Invitrogen) was placed in the lower chambers as chemoattractant or, alternatively, 48h rat astrocyte conditioned media. The chambers were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 24h. Non-migrating cancer cells on the upper surface of the inserts were removed by wiping with a cotton swab; migrating cells on the lower surface were fixed and stained with Insta Stain 3 Step (S&K Reagent, Denver, CO, USA). The invasive potential was quantified by counting the total number of cells on the lower surface of the inserts under a light microscope at 400X magnification. Three random visual fields were counted for each assay. Triplet was carried out in each invasion experiment.

#### *Rat inoculation*

Forty-day-old BD-IV rats were used. The animals were obtained from a colony maintained at Texas A& M University in accordance with institutional animal care guidelines. The syngeneic animals were inoculated with  $1 \times 10^4$  tumor cells in the left ventricle or alternatively in the mammary gland. Inoculation was performed on animals under Ketamine (87mg/kg, intramuscular injection) anesthesia. The animals were humanely euthanatized using Pentobarbital (150mg/kg, intraperitoneal injection) when showing clinical signs of discomfort. Complete necropsies were performed and tissues

sampled for histological evaluation. Six brain sections were done per animal and metastatic foci were counted.

#### *Orthotopic tumor growth assay*

Animals were inoculated in the mammary fat pad with 1X10<sup>4</sup> ENU1564-TIMP2 (n=3) or ENU1564 cells (n=3). Tumor size was determined at two time points by the use of a caliper. Tumor weight was determined at the time of death by complete dissection of mammary tumor. Six lung sections from each animal were evaluated histologically and metastatic foci were counted.

#### *Gel zymography*

Gelatin zymography was performed as described previously [32]. In brief, samples were electrophoresed on 10% (wt/vol) a polyacrylamide gel containing 0.1% (wt/vol) gelatin. After electrophoresis, the gel was washed twice for 30 min each in 2.5% (vol/vol) Triton X-100 at room temperature and then incubated in substrate reaction buffer (50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, 0.02% [wt/vol] NaN<sub>3</sub>, pH 8.0 at 25 °C) for 8–18 h at 37°C with gentle shaking. The gel was then stained with Coomassie Blue R250 in 10% (vol/vol) acetic acid and 30% (vol/vol) methanol for 1h and destained briefly in the same solution without dye. Proteolytic activity was detected by a clear band indicating the lysis of the substrate.

### *Western blotting*

Cells and tissue samples were homogenized in lysis buffer in a 1V: 10V dilution (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0), supplemented with a mixture of protease inhibitors. Samples were run on a 9-12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated one hour in blocking buffer (20 mM Tris-HCl buffered saline containing 5% nonfat milk powder and 0.1% Tween 20). Blots were incubated at 4°C overnight with anti-MMP2 (1:2000), GFAP (1:1000) and phospho-ERK1/2 (1:1000) (MMP2 antibody was purchased from Santa Cruz Biotech, Santa Cruz, CA, USA; antibody for ERK1/2 from Cell Signaling, Beverly, MA, USA; GFAP from DAKO, Denmark), washed extensively and then incubated for one hour with a 1:5000 dilution of anti-primary antibody. After additional washes, the blots were incubated with chemiluminescent substrate, according the directions in the kit (SuperSignal® West Pico, Pierce, Rockford, IL, USA).

### *Immunohistochemistry*

Five-micron (5µm) paraffin-embedded sections were used. Deparaffinization, rehydration and antigen-retrieval were done by immersion of slides in DECLERE® (Cell Marque, Hot Springs, AR, USA) commercially available buffer in moist heat (pressure cooker) for 15 minutes. Potential non-specific binding sites were blocked with 5% normal goat or rabbit serum in PBS. After blocking, the sections were incubated with primary phospho-ERK1/2 antibody (1:100) and GFAP (1:800). After three five-minute washes in

PBS, the sections were incubated with either biotin-conjugated anti-rabbit or anti-goat IgG (Vector Laboratories, Burlingame, CA, USA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development. Counter-staining was done with diluted hematoxylin. Sections that were not incubated with primary antibody served as negative control.

#### *MAPK inhibition assay*

ERK1/2 mitogen-activated kinase (MAPK) inhibitor, PD98059 (EMD Biosciences, Inc. San Diego, CA, USA) was used. It is a selective and cell-permeable inhibitor of MAP kinase kinase (MEK) that acts by inhibiting the activation of MAP kinase and subsequent phosphorylation of MAP kinase substrates. It was used dissolved in DMSO in a 10mM solution. Fifty microliters of the solution are added to 5 ml culture media at 24h of the experiment. PD98059 was added to ENU1564 cells and ENU1564 cells incubated with astrocyte-conditioned media.

#### *Statistical analysis*

Paired T-student tests were performed for all parameters including densitometry values obtained from gel zymography and Western blotting-autoradiograph analysis using Flour S MultiImager® technology from Biorad (Hercules, CA, USA). Differences were considered statistically significant when  $p < 0.05$ .

## Results

### *In vitro and in vivo TIMP2 expression*

Because we observed increase of MMP2 activity/expression in breast cancer brain metastases in our model, we over expressed tissue inhibitor of MMP2 (TIMP2) in the ENU1564 cell line to determine if MMP2 plays a role in the brain metastatic process. After selecting cell culture clones that expressed TIMP2, cell extracts were prepared, and WB was performed on the cell culture extracts (**Figure 20a**). The clone with higher expression of TIMP2 was selected for in vivo inoculation. To confirm the increased expression of TIMP2 in vivo, tumor cell extracts were obtained from the tumor masses derived from mammary fat pad inoculation. Tumors derived from ENU1564-TIMP2 cell lines had significantly higher levels of TIMP2 protein expression when compared with controls inoculated with ENU1564 cells ( $p < 0.001$ ) (**Figure 20b & 20c**).

### *Effect of TIMP2 transfection on orthotopic tumor growth*

In order to determine if MMP2 has a role in in vivo tumor growth at the primary mammary gland site, we inoculated ENU1564-TIMP2 cells into the mammary pad of BD-IV rats. We observed, at two different time points, that tumors derived from ENU1564 cells were significantly larger than tumors derived from ENU1564-TIMP2 ( $p < 0.001$  and  $p < 0.05$  in the two different time points, day 32 and day 42 post inoculation) (**Figure 21**).

Tumor weights were obtained at the time of sacrifice (day 42 post inoculation). The tumors from control rats, originated from ENU1564 inoculation, were heavier than tumors

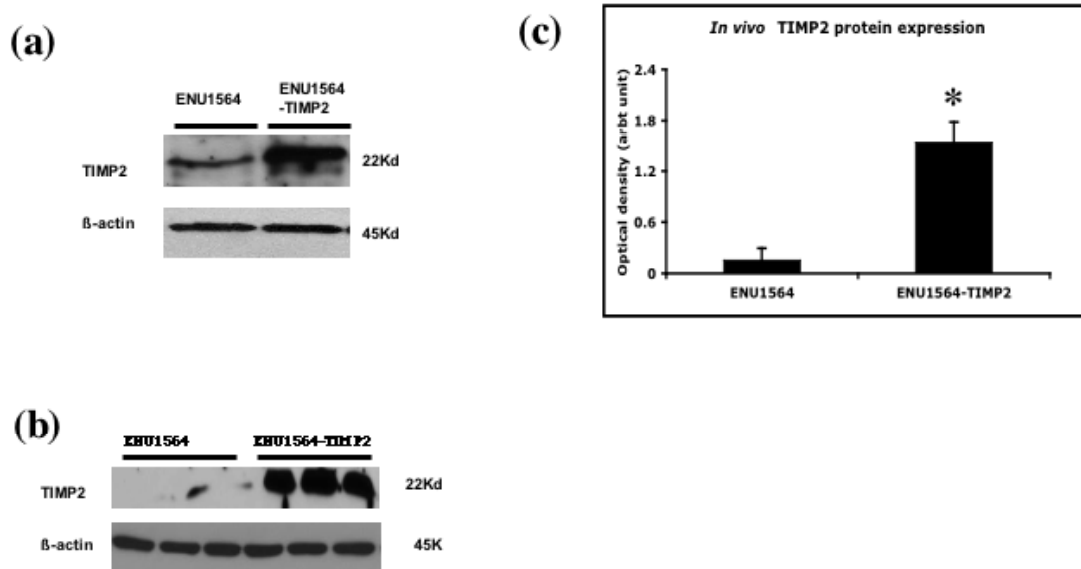


Figure 20. Establishment of TIMP2 overexpression in cancer cells. (a) *In vitro* TIMP2 expression in ENU1564-TIMP2 compared with ENU 1564 control cells. (b) *In vivo* expression of TIMP2 in orthotopic tumors derived from inoculation with ENU 1564-TIMP2 vs ENU 1564 control cells. The membranes were stripped and re-probed with  $\beta$ -actin antibody to confirm equal protein loading and transfer. (c) Quantitative analysis of TIMP2 *in vivo* expression was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. \*, for statistically significant when  $p < 0.05$ .



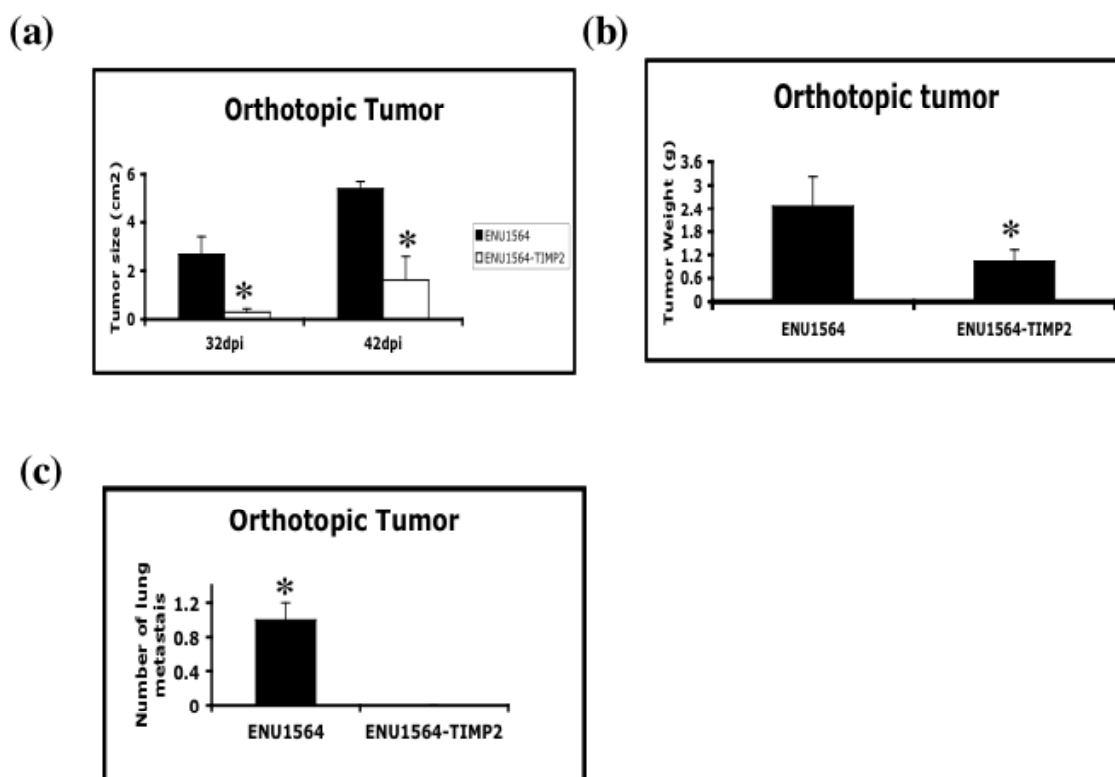


Figure 21. Effects of TIMP2 overexpression on orthotopic tumor development originated from inoculation with ENU1564-TIMP2 vs ENU1564 control cells in the mammary fat pad. (a) Orthotopic tumor size determined in two different days. (b) Orthotopic tumor weight determined at time of animals' sacrifice. (c) Number of lung metastases originated from orthotopic mammary tumors. (\*) for statistically significant when  $p < 0.05$ .

from animals inoculated with ENU1564-TIMP2 cells ( $p < 0.05$ ). Additionally, histological evaluation of lungs from both groups revealed that only animals from the control group developed lung metastases.

#### *In vitro invasive potential of cancer cells*

To determine whether TIMP2 overexpression has any influence on in vitro ENU1564 invasive potential, we used a Matrigel-based invasion assay. Boyden chamber chemoinvasion analysis showed that a significantly smaller number ( $p < 0.001$ ) of ENU1564-TIMP2 cells invaded the Matrigel when compared with ENU1564 cells. (**Figure 22**).

#### *In vivo assessment of MMP2 expression and activity*

Because we did not observe development of brain metastases in any of the animals inoculated with ENU1564-TIMP2 cells, we used material collected from orthotopic mammary tumors developed from inoculation of both ENU1564 and ENU1564-TIMP2 cells to evaluate in vivo change in expression and activity of MMP2. WB evaluation revealed non-significant difference in levels of MMP2 protein ( $p > 0.1$ ) (**Figure 23a&b**). Gel zymography evaluation revealed significantly higher MMP2 activity in samples obtained from animals inoculated with ENU1564 cells when compared with animals

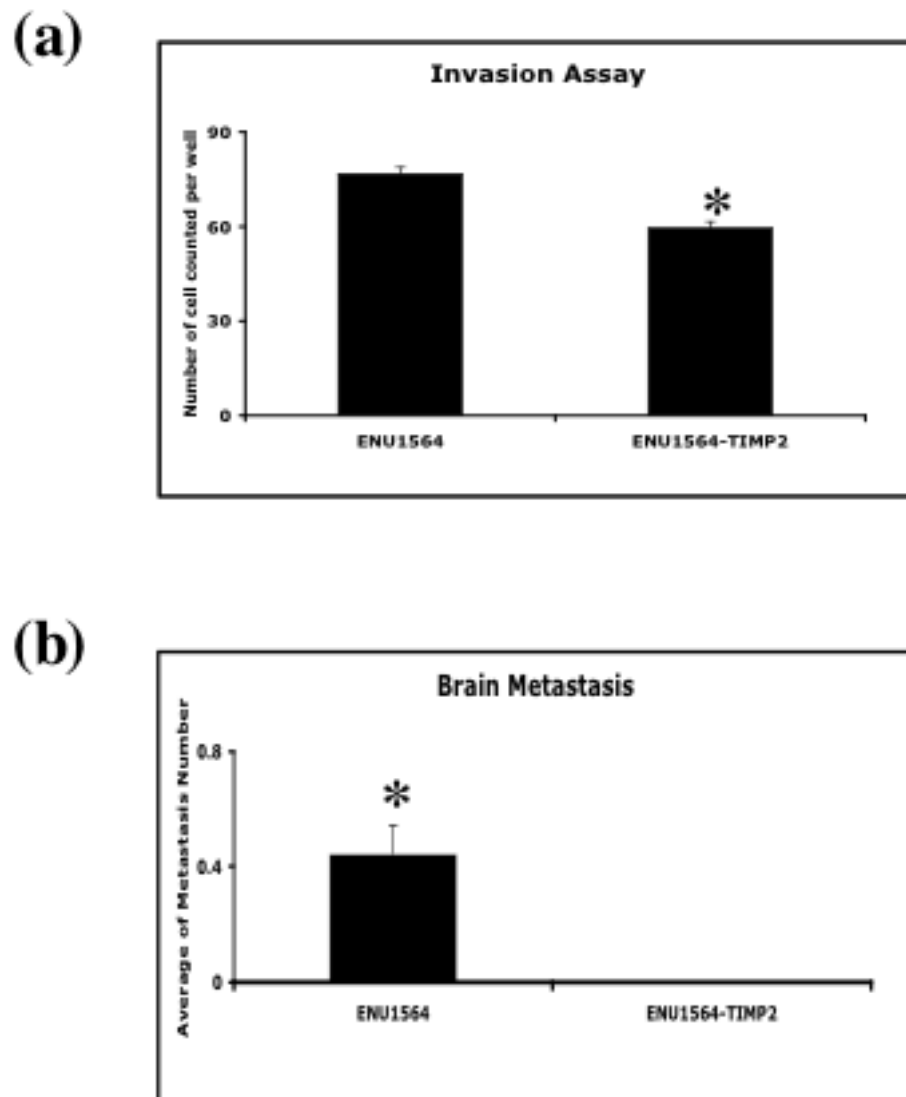


Figure 22. Metastatic potential of ENU1564 vs ENU1564-TIMP2. (a) *In vitro* invasion chamber assay for ENU1564 cells was performed as described in material and methods. The results shown in the histogram are the mean  $\pm$  standard deviation of two individual experiments run in triplicate. (b) Effects of TIMP2 overexpression in brain metastatic tumor development. (\*) for statistically significant when  $p < 0.05$ .

health of animals in the control group, all animals were sacrificed. Gross evaluation did not reveal any significant changes in the central nervous system. Upon histological evaluation 44.4% of the animals in the control group had brain metastases. Brain metastases were not detected in any of the animals in the group inoculated with ENU1564-TIMP2 cells ( $p<0.001$ ) (**Figure 22**).

*Effect of TIMP2 transfection in brain metastasis development*

To determine if MMP2 plays a role on breast cancer brain metastases development, we used cells that have high in vivo expression of tissue inhibitor of MMP2. We inoculated ENU1564-TIMP2 cells in the left ventricle. In parallel we inoculated control animals with ENU1564 cells. Five weeks after inoculation, due to deterioration of inoculated with ENU1564-TIMP2 cells ( $p<0.05$ ) (**Figure 23c&d**).

*In vivo and in vitro astrocytic activity*

Astrocytes play an important role in the development of breast cancer brain metastases [1, 25]. We observed previously that breast cancer brain metastases induce a marked astrocytic response. To better understand the role of astrocytes in the development of brain metastases of breast cancer in our model, we evaluated GFAP (glial fibrillary protein-astrocytic marker) reactivity in brain sections of animal with metastases by IHC. We observed a marked increase of immunohistochemical staining of GFAP in reactive

astrocytes around brain metastatic foci when compared with normal brain (**Figure 24a**). To confirm the IHC results, we performed WB analysis for GFAP protein in samples obtained from dissected brain frozen specimens. Additionally, we observed that tumor tissue expresses higher levels of GFAP protein when compared with control brains of animals

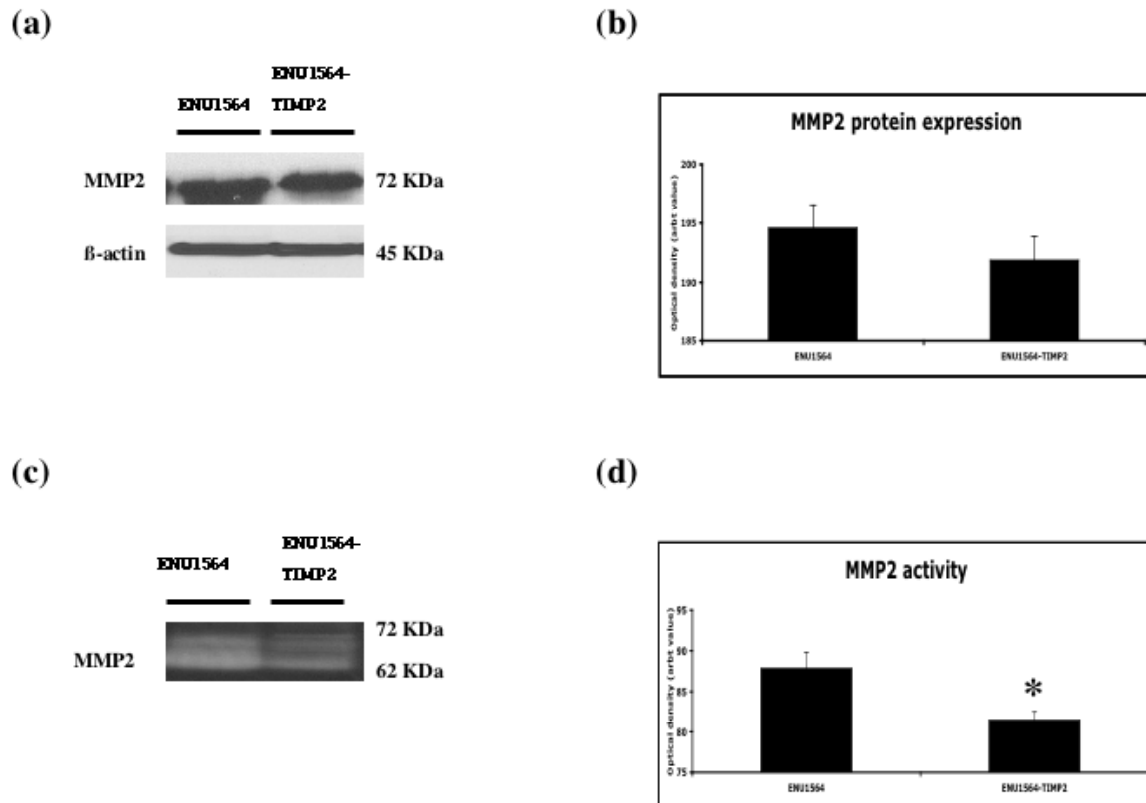


Figure 23. MMP2 enzymatic activity and MMP2 protein expression in orthotopic tumors originated from inoculation with ENU1564-TIMP2 vs ENU1564 control cells. (a) Evaluation of protein expression by Western blotting. The membrane was stripped and re-probed with  $\beta$ -actin antibody to confirm equal loading. (b) Quantitative analysis of MMP2 expression was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (c) Evaluation of MMP2, activity by gel zymography. (d) Quantitative analysis of MMP2 activity was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (\*) for statistically significant when  $p < 0.05$ .

free of tumor ( $p < 0.05$ ) (**Figure 24b & c**). The use of 48h-rat astrocyte-conditioned media as a chemoattractant revealed an increase in invasive behavior of ENU1564 cells ( $p < 0.001$ ) that was reduced in ENU1564-TIMP2 transfected cells ( $p < 0.001$ ) (**Figure 24d**). To determine if astrocytes have a role in MMP2 expression in ENU1564 cells, we treated these cells with 48h conditioned rat-astrocyte media. WB evaluation of MMP2 expression in ENU1564 cells revealed that these express low levels of MMP2. When incubated with astrocyte-conditioned media we observed an increased expression of MMP2 protein by WB evaluation ( $p < 0.05$ ).

#### *ERK1/2 pathway-mediated MMP2 expression*

We observed a relationship between MMP2 expression/activity in the development of BC brain metastases. We found that astrocytes affect MMP2 expression in ENU1564 cells. The MAPK has been associated with the metastatic cascade, MMP2 activity and astrocytic factors [23, 24, 26]. To determine if there is any correlation between brain metastases and the main components of the MAPK pathway in our model, we performed IHC analysis for ERK1/2, p38 and JNK. Neoplastic epithelial cells were positive for phosphorylated-ERK1/2 and staining was more intense at the periphery of the neoplastic lesions (**Figure 25a&b**). Neoplastic cells did not stain with p38 and JNK antibodies. To confirm the IHC results, we performed WB analysis for phosphorylated-ERK1/2 protein

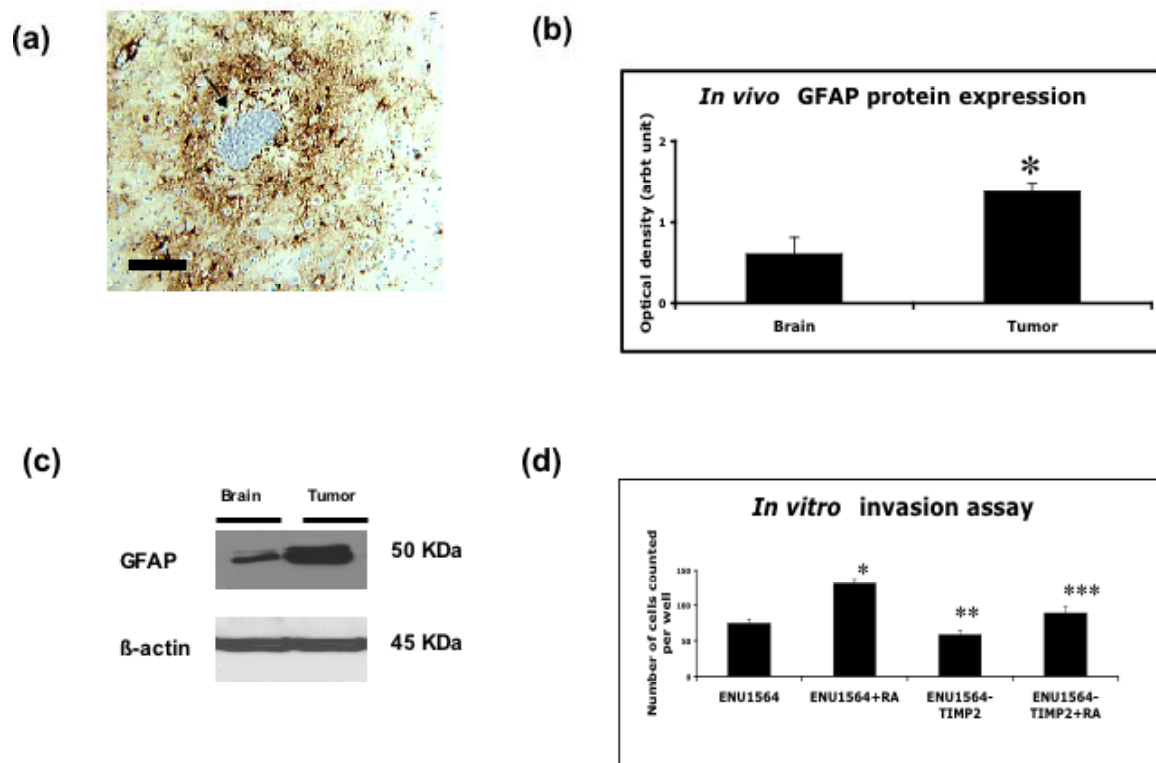


Figure 24. *In vivo* and *in vitro* astrocyte activity (a) Immunohistochemical staining (brown) of GFAP (glial fibrillary acidic protein) protein around metastatic foci. Arrows indicate metastatic foci. Bars indicate 100 μm. (b) *In vitro* invasion chamber assay for ENU1564 cells was performed as described in material and methods. The results shown in the histogram are the mean  $\pm$  standard deviation of two individual experiments run in triplicate. \*, statistically significantly different from ENU1564 ( $p < 0.05$ ). \*\*, statistically significantly different from ENU1564 ( $p < 0.05$ ). \*\*\*, statistically significantly different from ENU1564-RA (rat astrocyte-conditioned media treated-ENU1564) ( $p < 0.05$ ). (c) *In vivo* evaluation of GFAP protein expression in brain metastatic foci by Western blotting. The membranes were stripped and re-probed with  $\beta$ -actin antibody to confirm equal protein loading and transfer. (d) Quantitative analysis of GFAP protein expression was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (\*) for statistically significant when  $p < 0.05$ .

in samples obtained from dissected frozen brain specimens. We observed that tumor tissue expresses higher levels of phosphorylated-ERK1/2 protein when compared with control

brains of tumor free animals ( $p<0.05$ ) (**Figure 25c&d**). To evaluate the role of phosphorylated-ERK1/2 in the expression of MMP2 in ENU1564 cancer cells treated with 48h astrocyte-conditioned media we used ERK1/2-MAPK inhibitor, PD98059. We observed that phosphorylated-ERK1/2 was up regulated in the breast cancer cell line when treated with astrocyte-conditioned media ( $p<0.05$ ). The analysis of cell extract protein expression after treatment with PD98059 revealed a significant decrease of MMP2 protein expression ( $p<0.05$ ) as well as decrease in ERK1/2 expression ( $p<0.001$ ) (**Figure 26**).

## Discussion

The metastatic rodent models for breast cancer described to date study mostly nodal, pulmonary and bone metastases. In most of these studies, brain metastases occur as a non-predictable event and only sporadically [33, 34]. In a previous study using a rat syngeneic model for breast cancer brain metastases, we determined that breast cancer brain



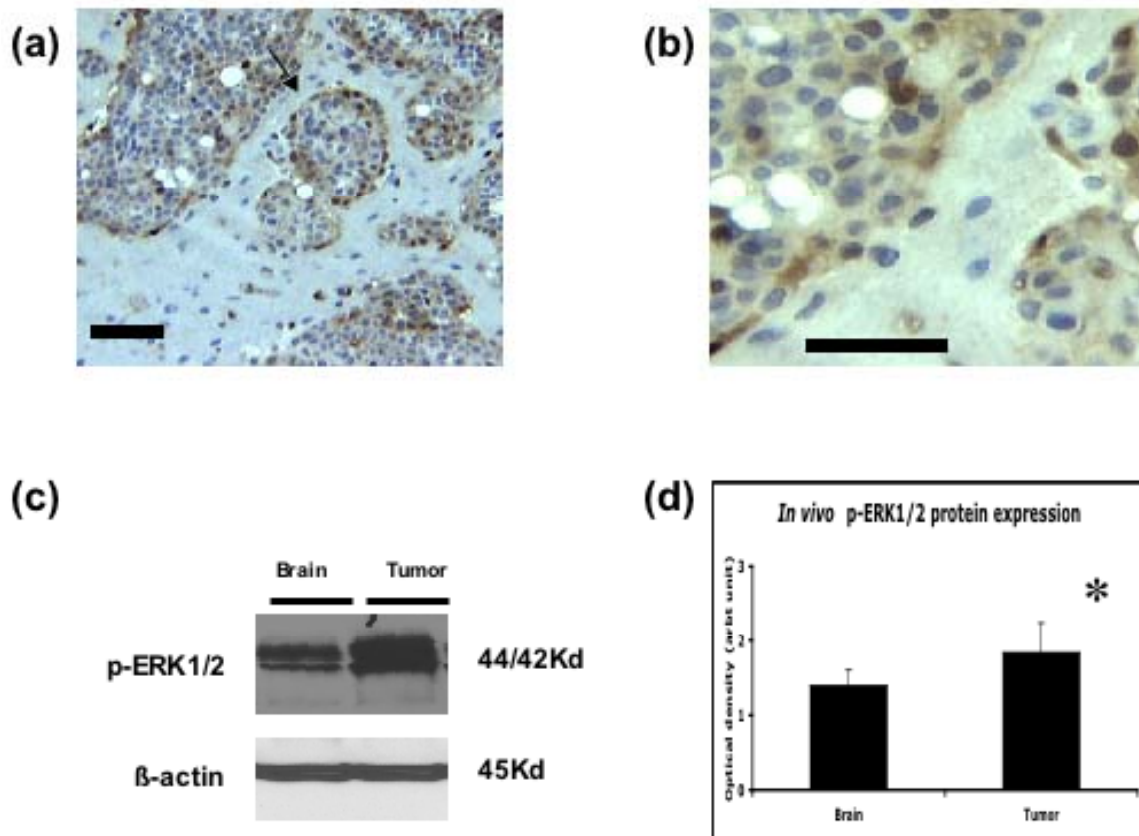


Figure 25. *In vivo* and *in vitro* phosphorylated-ERK1/2 protein expression in breast cancer brain metastatic foci. (a)&(b) Immunohistochemical staining (brown) of phosphorylated-ERK 1/2 protein. Arrows indicate metastatic foci. Bars indicate 100µm. (b) *In vivo* evaluation of phosphorylated-ERK 1/2 protein expression in brain metastatic foci by Western blotting. The membranes were stripped and re-probed with β-actin antibody to confirm equal protein loading and transfer. (c) Quantitative analysis of phosphorylated-ERK1/2 protein expression was determined by densitometry. The results shown in the histogram are the mean ± standard deviation from three control and tree tumor samples. (\*) for statistically significant when  $p < 0.05$ .

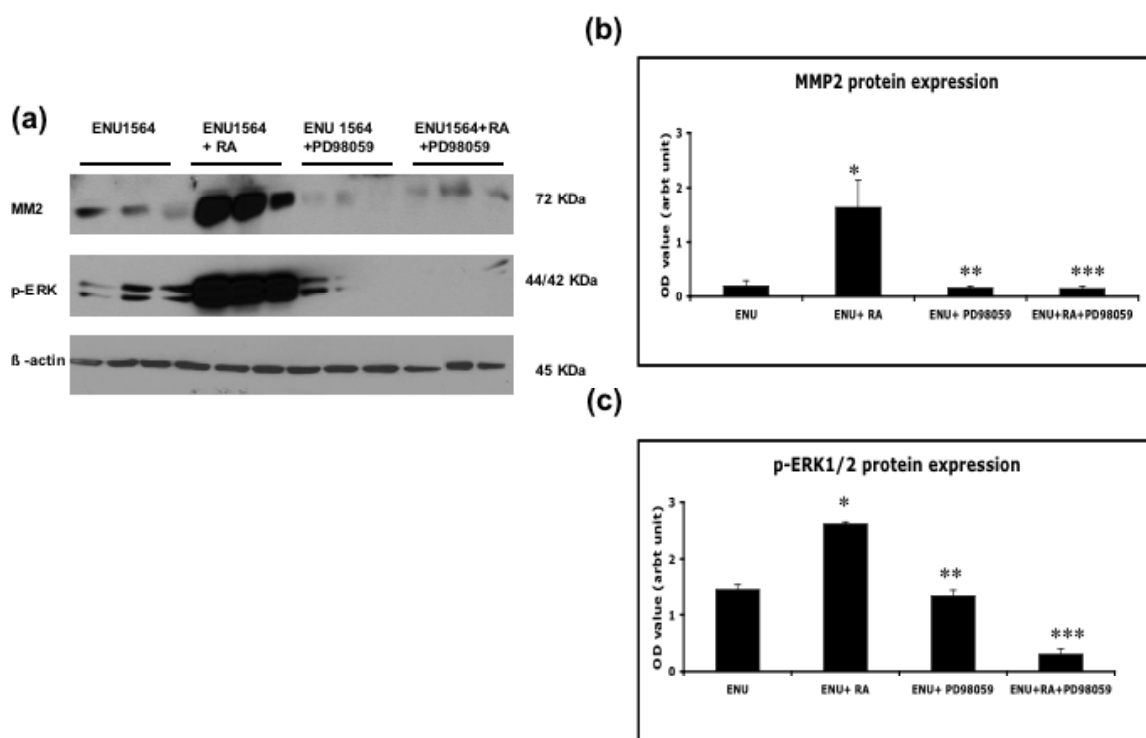


Figure 26. *In vitro* ERK1/2 pathway-mediated MMP2 regulation. (a) Evaluation of phosphorylated-ERK1/2 and MMP2 protein expression by Western blotting. The membranes were striped and re-probed with  $\beta$ -actin antibody to confirm equal protein loading and transfer. (b) Quantitative analysis of phosphorylated-ERK1/2 expression was determined by densitometry. (c) Quantitative analysis MMP2 expression was determined by densitometry. The results shown in the histograms are the mean  $\pm$  standard deviation from three individual experiments run in triplicate. \*, statistically significantly different from ENU1564 ( $p < 0.05$ ). \*\*, statistically significantly different from ENU1564 ( $p < 0.05$ ). \*\*\*, statistically significantly different from ENU1564-RA (rat astrocyte-conditioned media treated-ENU1564) ( $p < 0.05$ ).

metastases significantly express higher levels of MMP2 and have increased MMP2 activity (in print). These results suggest that this proteinase may play a role in the development of breast cancer brain metastases.

Here, we report that inhibition of MMP2 by overexpression of tissue inhibitor of MMP2 (TIMP2) causes reduction in metastases of breast cancer to the brain. Furthermore, we intend to investigate a possible role of astrocytes in MAPK signaling on MMP2 expression.

MMP2 is believed to play an important role in breast cancer invasion and metastases [9]. MMP2 overexpression and activation have been associated with the invasive potential of human tumors. Active MMP2 was detected more frequently in malignant than benign breast carcinomas [6]. A few studies are available about the role of MMPs in breast cancer metastases. However, fewer have characterized the expression of these molecules in breast-to-brain cancer metastases. It is important to determine if MMPs have different effects/roles in the development of metastases in different organs because this may contribute to understanding why BC cells metastasize to preferential organs. Here, we concentrate on the metastatic process of BC to the brain.

TIMP2 expression is important for MMP2 activity. In BC patients it has been correlated with poor prognosis [4, 46]. We observed that transfection of breast cancer cells with TIMP2 causes in vitro TIMP2 overexpression that is associated with decrease in in vitro cancer cell invasive behavior. When these transfected cells are inoculated in vivo, there is a marked increase in TIMP2 expression, which is associated with marked variations of cancer cell biological behavior. TIMP2 overexpression decreases orthotopic

tumor growth, size and weight; and also influences the metastatic behavior of orthotopic tumors. None of the animals inoculated orthotopically, with ENU1564-TIMP2 cells developed lung metastases, compared with development of lung metastases in all animals inoculated with ENU1564 cells. Additionally, and in concurrence with these results, none of the animals inoculated with ENU1564-TIMP2 cells developed brain metastases when inoculated via the left ventricle. These results suggest that TIMP2 expression decreases the metastatic brain behavior of BC cancer cells in this model. In order to determine if there was any variation in in vivo expression and activity of MMP2, and because we did not detect development of brain metastases in any of the animals inoculated with ENU1564-TIMP2 cells, we analyzed material collected from orthotopic mammary tumors developed from animals inoculated with ENU1564 and ENU1564-TIMP2 cells. As expected, no significant variation was observed in the levels of MMP2 protein expression, because TIMP2 has no reported influence on MMP protein expression. However, tumors derived from animals inoculated with ENU1564 cells had higher MMP2 activity when compared with tumors originated from animals inoculated with ENU1564-TIMP2 cells. These results suggest that TIMP2 overexpression effectively decreases MMP2 activity and that MMP2 is important in the biological brain metastatic behavior of cancer cells. Our results are in concurrence with results of previous studies. Cell transfection with TIMP2 in in vivo models decreases not only tumor growth but also metastatic potential [39, 40]. Experimental TIMP2 overexpression is related to decreased node and pulmonary metastases in bladder cancer [52], adenovirus-mediated TIMP2 delivery decreases the

numbers of pulmonary metastases [49], retroviral delivery of TIMP2 decreases BC invasion and metastases [91].

Astrocytes may play an important role in the development of brain metastases, as they have been shown to respond to extracellular stimuli by producing many cytokines and growth factors that can modulate tumor cell proliferation, growth and/or metastases. Cytokines produced by glial cells in vivo (such as IL6, tumor necrosis factor alpha and IGF 1) may contribute, in a paracrine manner, to the development of brain metastases by breast cancer cells [58, 61]. We reported previously that there is prominent astrocyte reaction associated with BC brain metastatic foci. We wanted to investigate if astrocytes play a role in the development of breast cancer brain metastases in our system and if this role is related to MMP2. Increased IHC stain of GFAP around brain metastatic foci when compared to brain tissue non-infiltrated by neoplasia suggests that astrocytes are associated with the development of BC brain metastases. Additionally, tumor samples express higher levels of GFAP protein when compared with controls. We also observed that the use of astrocyte-conditioned media as chemoattractant increases invasive behavior of both ENU1564 and ENU1564-TIMP2 cells. Rat astrocyte-conditioned media causes increased in vitro expression of MMP2 protein, but has no effect in in vitro MMP3 and/or MMP9 protein expression (data not shown). These results support that astrocytes are associated with MMP2 expression and that this may be related to cancer cell invasive phenotype.

Our previous studies demonstrated that expression and activity of MMP2 are increased in breast cancer brain metastases. Our present study shows that astrocytes may be involved in MMP2 up regulation. These points prompted us to investigate what signaling

pathway is involved in up regulation of MMP2 expression in ENU1564. MAPK pathway has been related to MMP activation and expression. MMP2 expression and activity are correlated with ERK phosphorylation [76]. Blockade of the ERK pathway by treatment with PD184352, a specific powerful inhibitor of MAPK/ERK kinase (MEK) suppressed expression of MMPs [72, 92]. In addition, activation of the ERK pathway in tumor cells is well correlated with cancer cell invasive and metastatic phenotype [77]. To determine if there is a correlation between brain metastases and the main components of the MAPK pathway, we performed IHC analysis for several components of the MAPK pathway phosphorylated ERK1/2, p38 and JNK. Neoplastic epithelial cells are positive for phosphorylated ERK1/2 and staining is more intense at the periphery of the brain metastatic foci. Since these peripheral tumor cells correspond to the invasive front of the tumor foci, these results suggest that phosphorylated ERK1/2 may be involved in brain metastases development. No staining was observed with p38 and JNK (data not shown). Additionally, we observed that brain tumor tissue expresses higher levels of phosphorylated ERK1/2 protein. Also, increase of phosphorylated ERK1/2 protein in ENU1564 cells after treatment with astrocyte-conditioned media was correlated with increased expression of MMP2 in these cancer cells. When we treated ENU1564 cells (with or without astrocyte conditioned media) with an ERK1/2 inhibitor (PD98059) we observed a significant decrease in MMP2 protein expression, as well as the expected decrease in phosphorylated ERK1/2 expression. These results strongly support that MMP2 expression is associated with phosphorylated ERK1/2 expression and is regulated, at least in part, by factors produced by astrocytes. Future studies will be needed to determine the specific

astrocytic factors that are associated with MMP2 expression within the CNS that are mediated through ERK1/2 signaling pathway.

We conclude that MMP2 is important in the process of establishment of breast cancer brain metastases. Additionally, we have evidence of the importance of astrocytes within the BC brain metastatic cascade and their possible correlation with MMP2 expression via ERK1/2-MAPK pathway.

## **CHAPTER IV**

### **EFFECTS OF MMP INHIBITION BY PD166793 IN THE DEVELOPMENT OF BREAST LUNG METASTASIS IN A RAT MODEL**

#### **Introduction**

A suitable tumor environment is important for the development of metastatic tumor cells [80]. Many theories have been developed to study and understand metastatic behavior. Factors such as molecular and genetic characteristics of neoplastic cell and biological environment are considered determinant in the metastatic process [9]. We aim to study the role played by matrix metalloproteinases (MMPs) and their inhibitors in the development of BC lung metastasis.

MMPs are a broad family of zinc-dependent proteinases that play a key role in extracellular matrix degradation, and are implicated in numerous pathogenic processes [9]. Tumor cells are believed to secrete these matrix-degrading enzymes and/or induce host cells to produce them [39]. In addition to the degradation of the extracellular matrix (ECM), MMPs are suspected of having other roles in cancer development, such as angiogenesis, inhibition of apoptosis [6], and regulation of cell proliferation [3]. High serum concentration and over-expression of MMP2, MMP3 and MMP9 in breast cancer patients is related to poor prognosis, decreased survival, increased tumor size, increased invasiveness and metastatic behavior [9, 11, 12, 16, 18, 14, 39, 93, 94, 95]. Data from



experimental animal models also correlates MMP expression with invasive behavior and increased metastasis [39].

The lung is one of the most frequent sites for breast cancer metastasis. MMPs have been studied not only in the context of lung metastasis, but also as a factor in the development of metastasis of pulmonary tumors. Several MMP inhibitors are under consideration for clinical trial in patients with cancer [96]. It is important to understand organ specific metastatic mechanisms in order to increase drug efficacy.

Here, we intend to determine if inhibition of MMP is important to the development of pulmonary metastasis of breast cancer in a rodent syngeneic model. To do this we used a MMP synthetic inhibitor (PD166793), known to inhibit/decrease MMP2, 3 and 9 activities. We will further characterize the metastatic system evaluating if MMPs influence cancer cell proliferation, cancer cell death as well as the relationship of MMPs and tumor stroma development.

## **Materials and methods**

### *Tumor cell line*

The ENU1564 tumor cell line used in this study was developed in our laboratory and originated from an N-ethyl-N nitrosourea-induced mammary adenocarcinoma in a female Berlin-Druckrey IV (BD-IV) rat. This cell line is highly metastatic to lung, brain and bone tissues [80].

### *Rat inoculation*

Forty-day-old BD-IV rats were used. The animals were obtained from a colony maintained at Texas A&M University in accordance with institutional animal care guidelines. The syngeneic animals were inoculated with  $1 \times 10^4$  tumor cells in the left ventricle. Inoculation was performed on animals under Ketamine (87mg/kg, intramuscular injection) anesthesia. The animals were humanely euthanatized using Pentobarbital (150mg/kg, intraperitoneal injection) when showing clinical signs of discomfort. Complete necropsies were performed and tissues were sampled for histology evaluation.

### *Drug experiment*

PD166973 (S-2-4'-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) is a specific MMP inhibitor. Dr J. Peterson generously provided PD166973 (Pfizer Inc, Ann Harbor, MI). The experiment was conducted in BD-IV rats. Five control animals received a single intracardiac inoculation of  $1 \times 10^4$  tumor cells and daily treatment with vehicle only (DMSO). Five animals were inoculated with  $1 \times 10^4$  neoplastic cells and treated daily. We used the dose of 5mg/kg/day as recommended in the literature for rat studies [PL26]. The study had the duration of 24 days. Animals were sacrificed at the end of the study and lung, and body weights were collected. Lung/body weight and lung/brain weight ratios were determined. Lung samples were placed in 10%formalin for histological evaluation. Six sections of lung were evaluated per animal. The lung lesions were scored from 1-7

according to size, presence of cell death, abundance of stroma, mitotic index, nuclear pleomorphism and number of metastatic foci (**Table 4**).

**Table 4.** PD166793 experimental design.

30 day old BD IV rats	Control group N=5	Treated group n=5
PD166793 (compound)	IP injection of vehicle only	5mg/kg/24 day IP (dissolved with DMSO)
ENU 1564 breast cancer cell line	1x10 <sup>4</sup> cells IC on d=1	1x10 <sup>4</sup> cells IC on d=1

#### *Tumor collection*

Samples were collected immediately after animal's death and placed on powdered dry ice until completely frozen. Samples were then kept at -80oC. The samples from metastatic tissue were collected from the lung by dissection.

#### *Immunohistochemistry (ICH)*

Five-micron (5  $\mu$ m) paraffin-embedded sections and 12 $\mu$ m frozen sections were used. Deparaffinization, rehydration and antigen-retrieval were done by immersion of slides in DECLERE® (Cell Marque, Hot Springs, AR) commercially available buffer in moist heat (pressure cooker) for 15 minutes. Potential non-specific binding sites were

blocked with 5% normal goat or rabbit serum in PBS. After blocking, the sections were incubated with primary antibodies in dilutions of 1:200 for MMP2; 1:25 for MMP3 and -9 purchased from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA); 1:500 for PCNA and 1:300 for Vimentin (antibodies for PCNA and vimentin were purchased from DAKO, Dako Cytomation, Denmark). After three five-minute washes in PBS, the sections were then incubated with biotin-conjugated anti-rabbit and anti-goat IgG (Vector Laboratories, Burlingame, CA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development. Counter-staining was done with diluted hematoxylin. Sections that were not incubated with primary antibody served as negative control.

#### *Western blotting (WB)*

The tissue was homogenized in lysis buffer in a 1V:10V dilution (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0, and 0.1% SDS), supplemented with a mixture of protease inhibitors. Samples were run on a 9-12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated one hour in blocking buffer (20 mM Tris-HCl buffered saline containing 5% nonfat milk powder and 0.1% Tween 20). Blots were incubated at 4°C overnight with anti-MMP2 (1:2000); MMP3 (1:500); MMP9 (1:1000) (antibodies from Santa Cruz Bitotech, Santa Cruz, CA), cleaved-caspase 3 (1:1000) (antibody form Cell Signaling, Beverly, MA) and washed extensively and then incubated for one hour with a 1:5000 dilution of anti-primary antibody. After additional washes, the blots were incubated with chemiluminescent

substrate, according the directions in the kit (SuperSignal ® West Pico, Pierce, Rockford, IL).

#### *Gel zymography*

Gelatin zymography was performed as described previously [28]. In brief, samples were electrophoresed on 10% (wt/vol) polyacrylamide gels containing 0.1% (wt/vol) gelatin and Ready Gel Zymogram Gel 12%, casein (Biorad, Hercules, CA). After electrophoresis, the gels were washed twice for 30 min each in 2.5% (vol/vol) Triton X-100 at room temperature and then incubated in substrate reaction buffer (50 mM Tris-HCl, 5 mM CaCl<sub>2</sub>, 0.02% [wt/vol] NaN<sub>3</sub>, pH 8.0) for 8 to 18 h at 37°C with gentle shaking. The gels were then stained with Coomassie Blue R250 in 10% (vol/vol) acetic acid and 30% (vol/vol) methanol for 1 to 2 h and destained briefly in the same solution without dye. Proteolytic activities were detected by clear bands indicating the lysis of the substrate.

#### *PCNA index evaluation*

The evaluation of PCNA index was performed according to the literature [97]. After IHC staining with PCNA antibody the number of cells with positive nuclear staining was evaluated in a total of 500 cells in five different high power fields (400X).

### *TUNEL assay*

Apoptotic cells were visualized by the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) technique using a NeuroTACS II in situ apoptosis detection kit (Trevigen, Gaithersburg, MD). The tumor sections fixed on glass slides were incubated with Proteinase K/Triton X-100 for 20 min at room temperature. TdT was used to catalyze the addition of biotin-conjugated dUTP to the 3'-OH ends of DNA fragments. The incorporated biotin was detected by streptavidin conjugated to horseradish peroxidase. The staining was done using DAB as chromogen and H<sub>2</sub>O<sub>2</sub> as substrate. The sections were counterstained with hematoxylin. The apoptotic index was determined by microscopic examination

### *Statistical analysis*

Student t-test was performed to evaluate all parameters including densitometry values obtained from Western blotting-autoradiographs and gel zymography analysis using Fluor S MultiImager® technology from Biorad (Hercules, CA). Differences were considered statistically significant when  $p \leq 0.05$ .

## Results

### *MMP expression in lung metastasis*

We evaluated the expression of MMP2, MMP3 and MMP9 in lung metastasis using IHC. We observed that neoplastic cells have strong expression of these molecules diffusely in the cytoplasm (**Figure 27**). To confirm these results we performed WB in lung metastatic tissue. We observed no significant difference in the levels of MMP2, -3 and -9 in lung metastasis when compared with levels of MMP2 expression in normal lung (**Figure 28**).

### *Drug experiment*

PD166973 (S-2-4'-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) is a selective MMP inhibitor, known to decrease the activities of MMP2, -3 and -9. We observed previously that PD166973 decreases in vitro invasive behavior of ENU 1564 cells (Chapter II). We use this compound to determine if inhibition of these molecules will alter in vivo development of lung metastasis. We observed that the lung weight/body weight and lung weight/brain weight ratios were higher in non-treated animals compared with PD166973 treated animals ( $p \leq 0.24$  and  $P \leq 0.017$  respectively) (**Figures 28 & 29**).

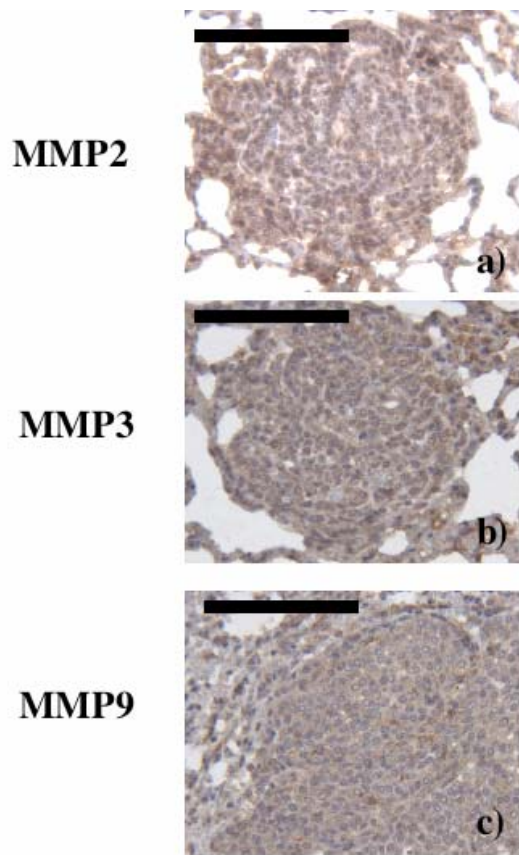


Figure 27. Expression of MMPs in lung metastasis (a) Immunohistochemical staining (brown) of MMP2 in the cytoplasm neoplastic cells in lung metastatic foci. (b) Immunohistochemical staining (brown) of MMP3 in the cytoplasm neoplastic cells in lung metastatic foci. (c) Immunohistochemical staining (brown) of MMP9 in the cytoplasm neoplastic cells in lung metastatic foci. Bars indicate 100 $\mu$ m.



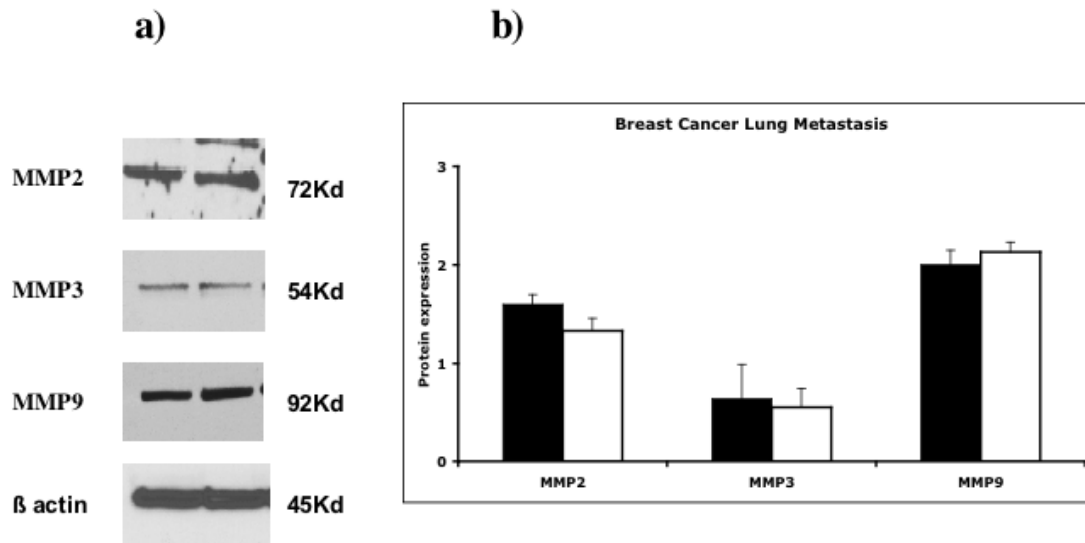


Figure 28. Expression of MMPs proteins in lung metastasis. (b) Expression of MMP2, -3 and -9 proteins in lung metastatic foci. The membranes were striped and re-probed with  $\beta$ -actin antibody to confirm equal loading (c) Quantitative analysis of MMP2; -3, -9 expression was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples.

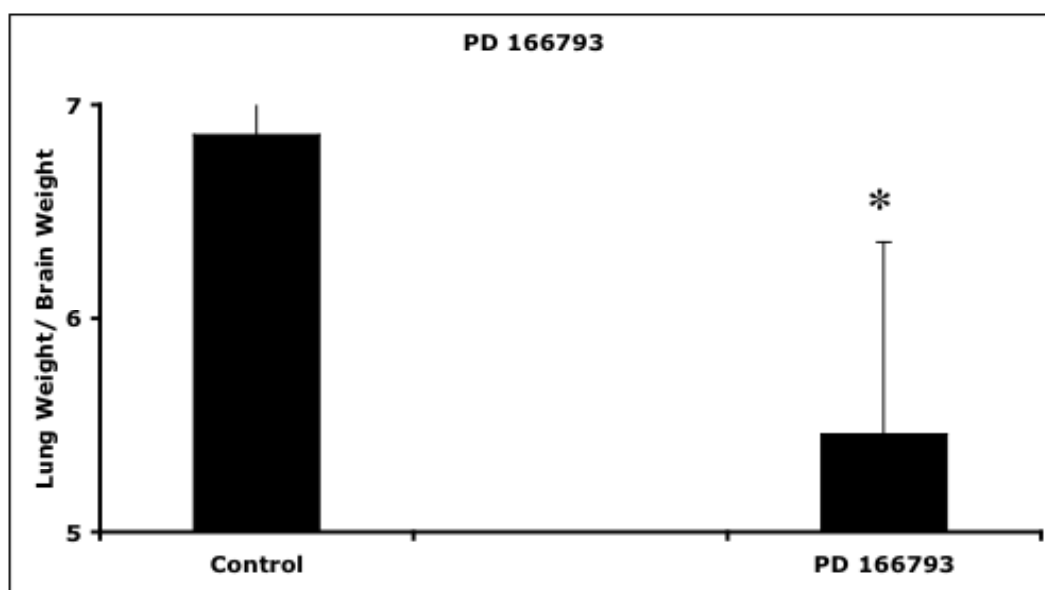


Figure 29. Effects of PD166793 in the development of lung metastasis. Evaluation of lung weight/brain weight ratio.(\*) for statistically significant when  $p \leq 0.05$ .

*In vivo assessment of MMP expression and activity*

We used material collected from metastatic lung tumors developed from inoculation of both animals treated with PD166973 vs animals that were vehicle treated, to evaluate in vivo change in expression and activity of MMP2, -3 and 9. WB evaluation revealed no significant difference in levels of MMP2, -3 and -9 protein expression ( $p > 0.1$ )

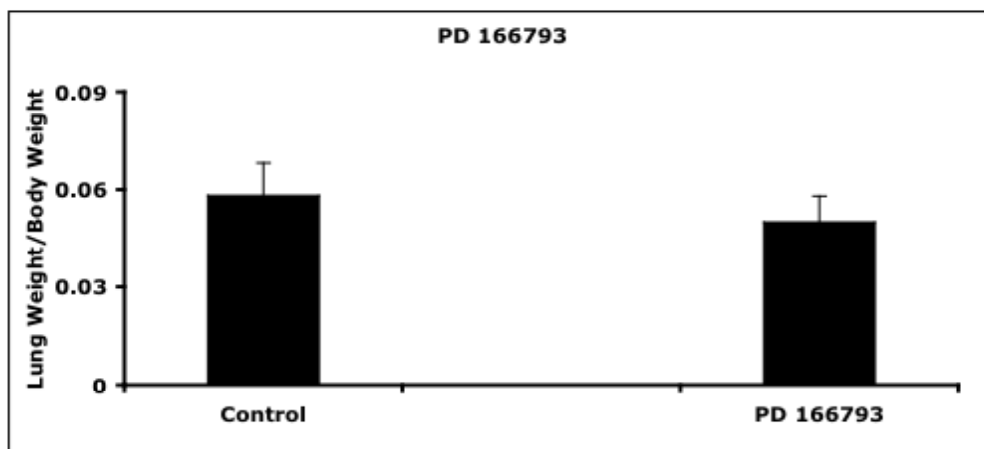


Figure 30 Effects of PD166793 in the development of lung metastasis. Evaluation of lung weight/body weight ratio.

**Table 5.** Effect of PD166793 in lung metastasis development.

	ENU1564	PD166793	Vehicle	p
		treated	treated	value
Mitotic index	7±0	6.4±0.54	7±0	0.035
Nuclear pleomorphism	6.33± 0.70	6.4±0.89	6.2±0.83	0.36
Stroma abundance	6.33±0.86	5.4±0.54	6.2±0.83	0.05
Cell death	6.11±1.16	5.8±0.83	6.2±0.83	0.235
PCNA index	43.5%	20.7%	47.9%	
Number of tumors	6±1	6.6±0.54	7±0	0.08
Tumor size	6.22±0.97	5.2±0.44	6.4±0.54	0.002

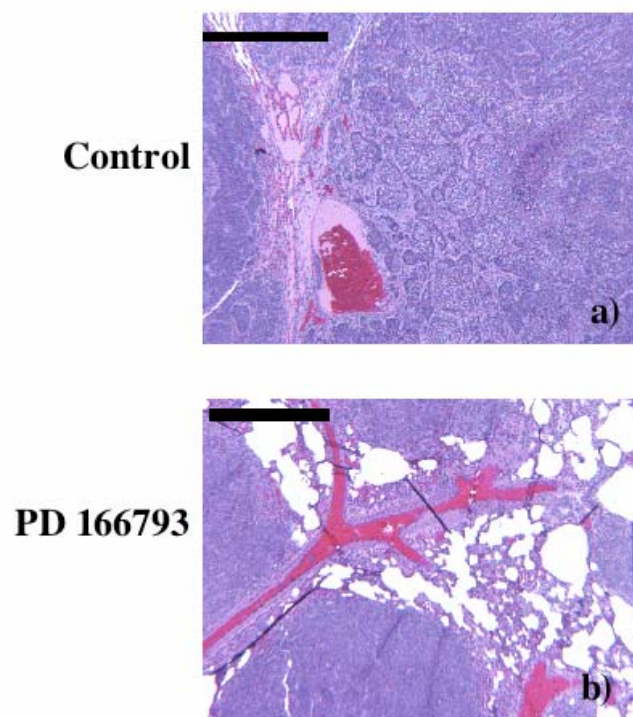


Figure 31. Effects of PD166793 in the development of lung metastasis. Histological evaluation of pulmonary metastasis size and number in animals treated with vehicle (a) and (b) animals treated with PD166793. Bars indicate 100µm.

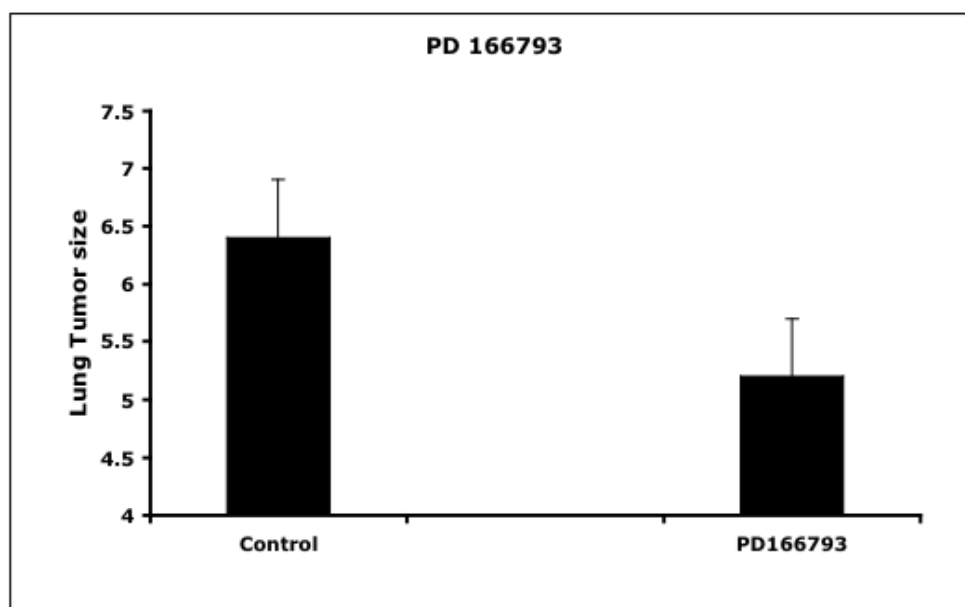


Figure 32. Effects of PD166793 in the development of lung metastasis. Evaluation of tumor size variation between animals treated with PD166793 and controls

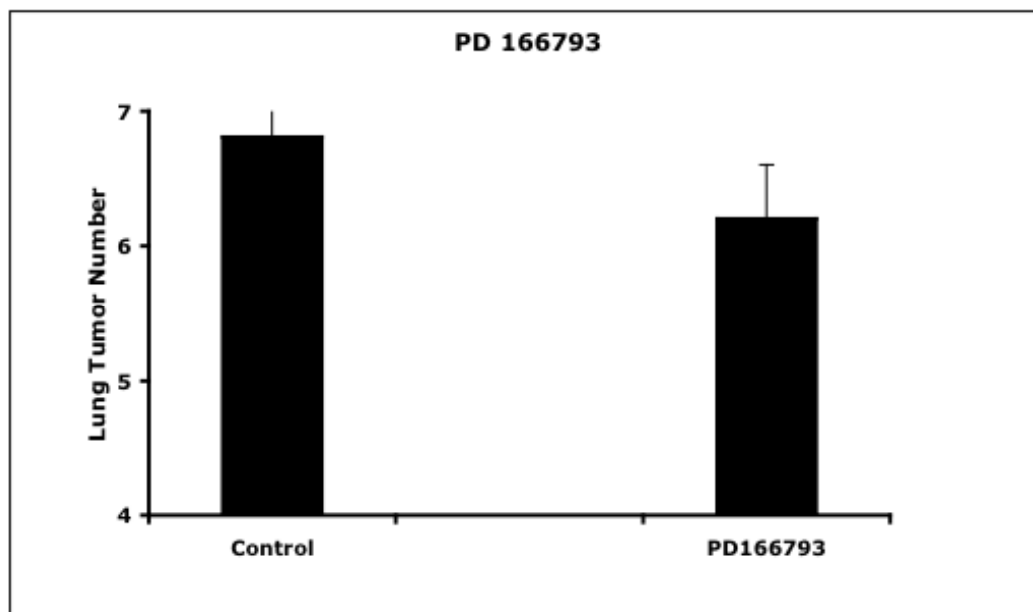


Figure 33. Effects of PD166793 in the development of lung metastasis. Evaluation tumor number variation between animals treated with PD166793 and controls.

Lung metastasis of animals treated with vehicle only, when compared with PD166793 treated animals were larger and present in higher numbers ( $p \leq 0.002$  and  $p \leq 0.08$ , respectively) (**Figure 31, 32 & 33**) (**Table 5**).

Gel zymography evaluation (**Figure 34**). revealed significant increased MMP9 activity in samples obtained from animals non treated with PD166793 cells when compared with animals treated with PD166793 ( $p \leq 0.04$ ) (**Figure 35**) as well as a decrease in MMMP2 and MMP3 activity in animals treated with PD166793.

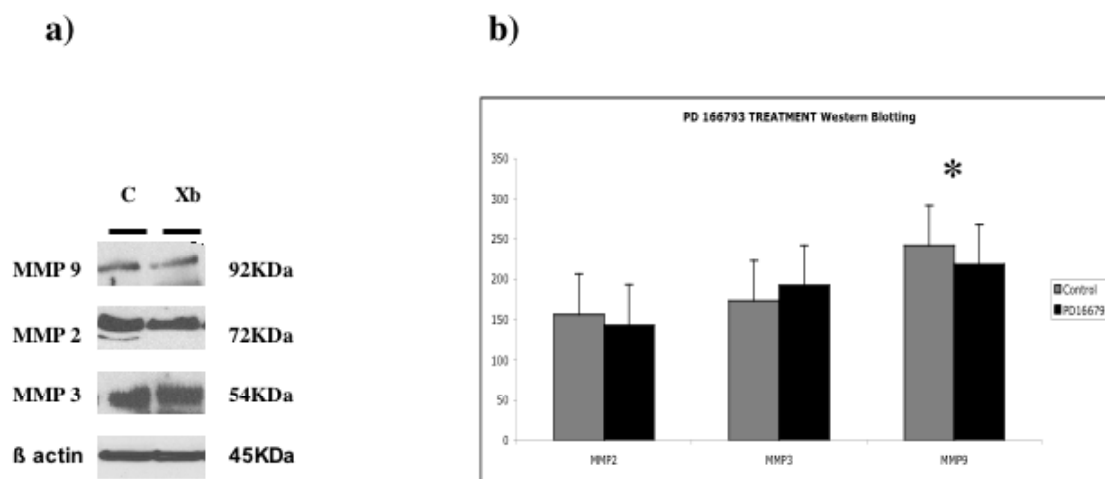


Figure 34. Effects of PD166793 in the development of lung metastasis. (b) Expression of MMP2, -3 and -9 in lung metastatic foci in animals treated with PD166793 and controls. The membranes were striped and re-probed with  $\beta$ -actin antibody to confirm equal loading (c) Quantitative analysis of MMP2, -3, -9 expression was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples.

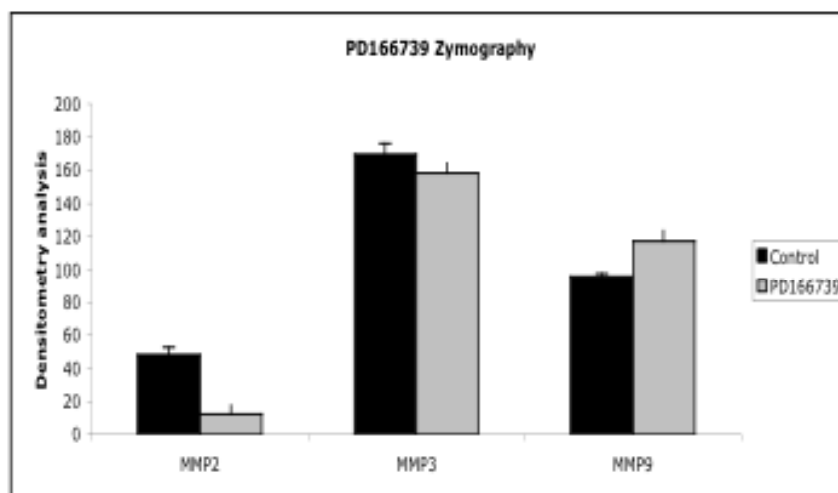


Figure 35. Effects of PD166793 in the MMP enzymatic activity. Quantitative analysis of MMP2, -3 and 9 activity was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (\*) for statistically significant when  $p \leq 0.05$ .

#### *Evaluation of cell proliferation*

In order to determine if there was an effect of MMP inhibition via PD166793 in tumor cell proliferation we evaluated histologically the mitotic index and nuclear pleomorphism (**Figure 36**).



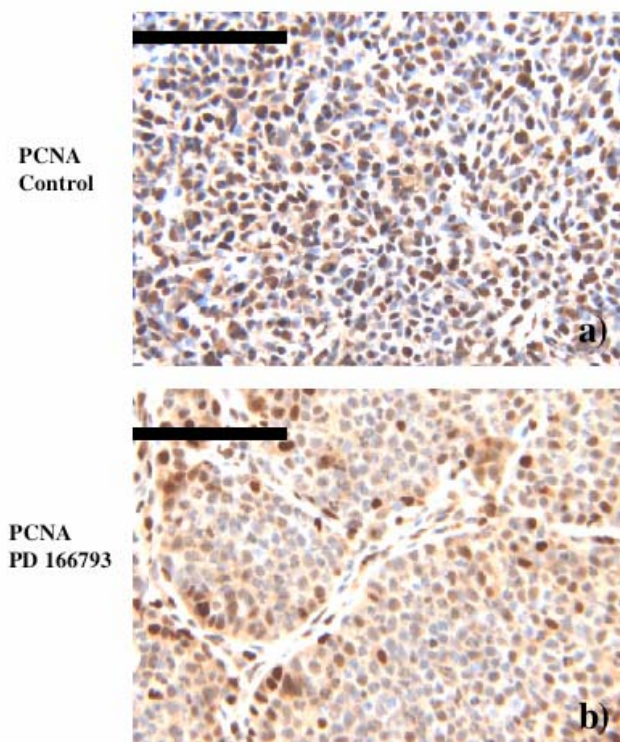


Figure 36. Effects of PD166793 in tumor cell proliferative index. Immunohistochemical staining (brown) of PCNA in the nucleus of neoplastic cells in lung metastatic foci. Bars indicate 100µm.

We confirmed these results by calculating the PCNA proliferative index. There was significantly higher mitotic index in non-PD166793 treated animals ( $p \leq 0.03$ ). There was also increased nuclear pleomorphism in non PD166793-treated animals ( $p \leq 0.36$ ). We observed that PCNA indexes were higher on lung metastasis originated from non-PD166793 treated animals (47.9%) when compared with PD166793 treated animals (20.7%).

#### *Evaluation of cell death*

To evaluate differences in cell death in the metastatic neoplastic foci we quantified the cell death phenotype by histological evaluation (**Figure 37**) and confirmed the evaluation by WB for cleaved-caspase 3. We observed that there was no significant difference in the amount of cell death in non-PD166793 treated animals when compared with PD166793 treated animals ( $p \leq 0.2$ ). There was also no significant difference in expression of cleaved-caspase3 protein in samples collected from non PD166793-treated animals vs samples collected from PD166793 animals ( $p \leq 0.49$ ) (**Figure 38**). Conversely TUNEL assay results did not show noticeable differences in apoptosis when samples of lung metastasis arising from PD166793 treated animals were compared to samples from non-PD166793 treated animals.

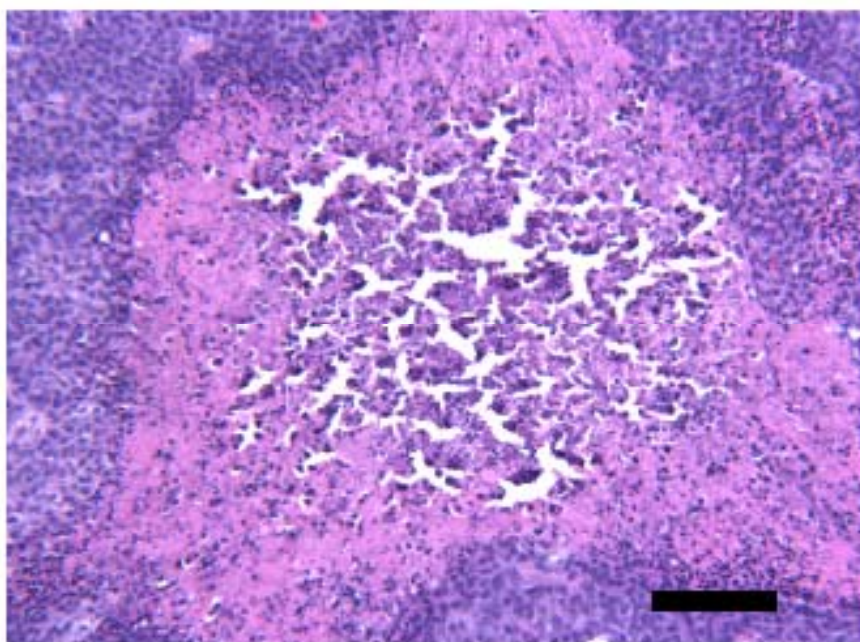
**Cell Death**

Figure 37. Effects of PD166793 in the development of lung metastasis. Histological evaluation of pulmonary metastasis cell death in treated with PD166793. Bars indicate 100 $\mu$ m.

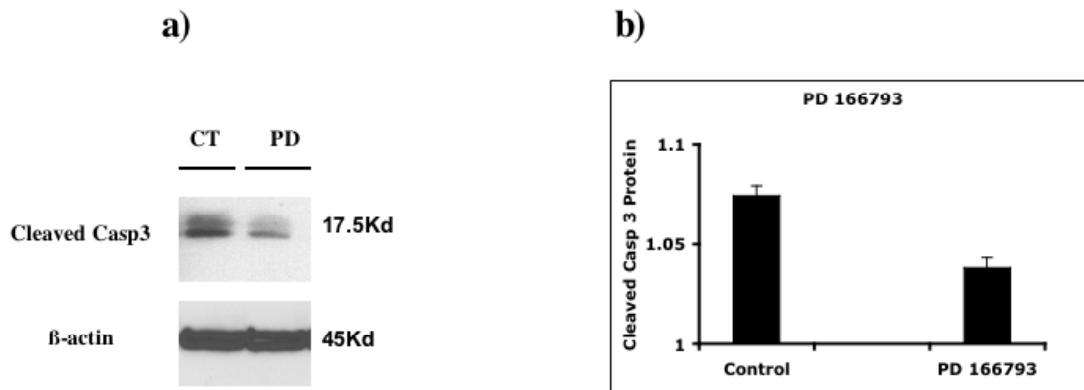


Figure 38 Effects of PD166793 in lung metastasis apoptosis. (a) Expression of cleaved-caspase 3 in lung metastatic foci in animals treated with PD166793 vs vehicle treated animals . The membranes were striped and re-probed with  $\beta$ -actin antibody to confirm equal loading (b) Quantitative analysis of cleaved-caspase3 was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples.

#### *Evaluation of cell stroma*

To evaluate differences in fibrous stroma in the metastatic neoplastic foci we quantified the amount of stroma by histological evaluation (**Figure 39**) and confirmed these results by Vimentin (mesenchymal cell marker) IHC staining (**Figure 40**). We observed that tumor stroma was more prominent in non-PD166793 treated animals ( $p \leq 0.05$ ) (**Figure 41**).

**Fibrous stroma**

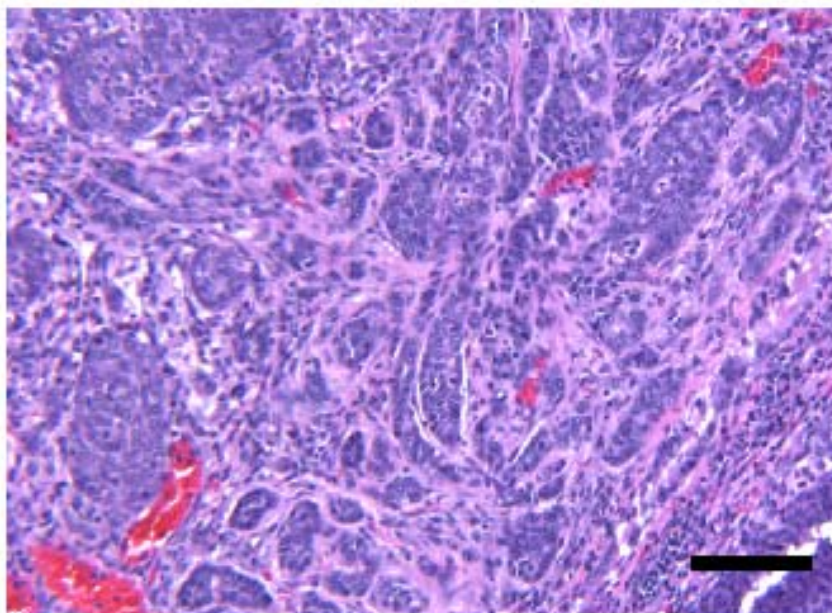


Figure 39. Effects of PD166793 in the development of lung metastasis. Histological evaluation of fibrous stroma in pulmonary metastasis in animals treated with PD166793. Bars indicate 100 $\mu$ m.

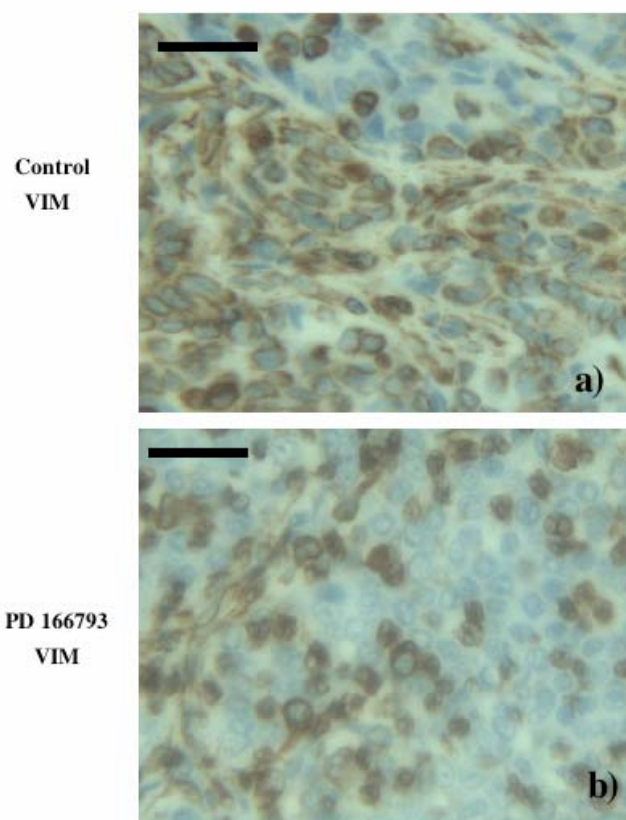


Figure 40. Effects of PD166793 in tumor stroma development. Immunohistochemical staining (brown) of vimentin in the cytoplasm of fibrous stroma cells in lung metastatic foci. Bars indicate 100µm.

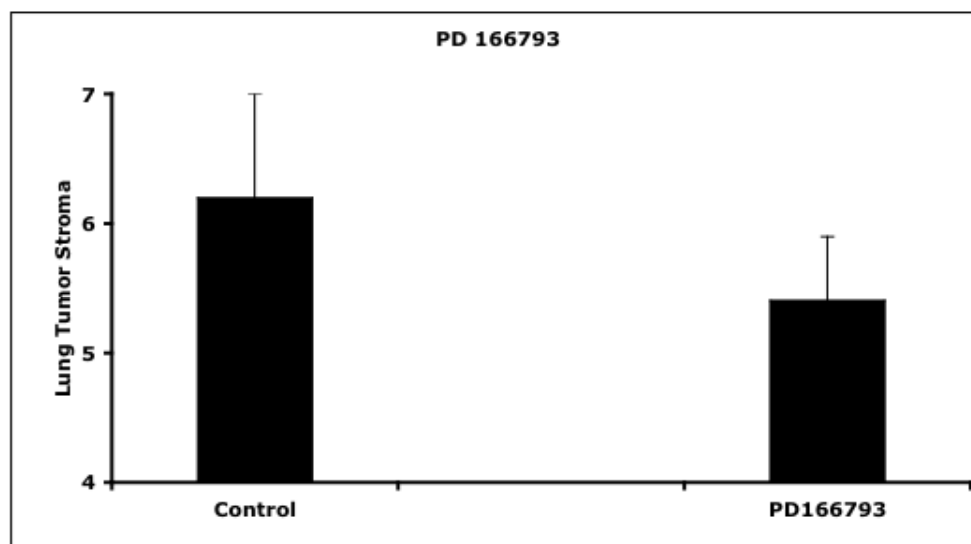


Figure 41. Effects of PD166793 in the development of lung metastasis. Evaluation of tumor stroma variation between animals treated with PD166793 and controls.

## Discussion

MMPs have been extensively studied in the context of breast cancer prognosis. Most of these studies have been performed on human tissue collected from patients diagnosed with breast cancer or in breast cancer cell lines. In this context, MMP2, -3 and -9 are thought to play an important role in breast cancer invasion and metastasis [18].

Our aim here is to study the relevance of MMPs in the development of BC lung metastasis. We evaluated the expression of MMP2, MMP3 and MMP9 in lung metastasis using IHC. We observed that neoplastic cells have strong expression of these molecules

diffusely in the cytoplasm. The evaluation of protein expression by western blot revealed that MMP protein levels studied were not increased in the lung metastasis when compared with to normal lung. This may be due to the fact that the lung is a environment physiologically rich in MMPs and because of that a marked difference is not noticeable. However, the strong expression of MMPs in the metastatic foci indicates that these molecules may still be important in the lung metastatic process. To determine if these molecules play a role in the development of metastasis we decided to inhibit MMP expression by use of an MMP synthetic inhibitor, PD166793.

Several synthetic MMP inhibitors are thought to inhibit both primary tumor invasion and tumor metastatic behavior. TN6b decreases the number of lung metastatic to lymph node [98]. BAY12-9566 decreases the number and volume of lung metastasis [99]. FYK1388 decreases lung metastasis of fibrossarcoma [100]. Prynomast decreases the incidence of lung cancer metastasis to the kidney [101].

Because MMP have been associated with lung metastasis development [102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112] , we used PD166973 (S-2-4'-bromobiphenil-4-sulfonylamino-3 methyl-butyric acid) that is a specific MMP inhibitor and unlike first generation of MMP inhibitors do not inhibit other metalloproteinases like TNF-alpha-convertase. This compound, known to decrease the activity of MMP2, -3 and -9, was used to determine if inhibition of these molecules will alter development of lung metastasis in treated compared to control animals. Lung weight/body weight and lung weight/brain weight ratios evaluation are highly suggestive that MMP inhibition caused decreased lung metastasis development. Additionally reduction in number and size of lung metastasis was



also observed. We therefore conclude that in our model MMPs play a role in the establishment of metastatic breast cancer to the lung. To evaluate in vivo variations in MMP activity and protein expression associated with PD166793 treatment we analyzed protein extracts derived from lung metastasis. No variation in the protein levels of MMP2, -3 and -9 was observed. This is due to the fact that PD166793 acts chiefly on MMP activity and not on MMP expression. Although we observed a decrease in MMP2 and MMP3 activities in animals treated with PD166793 these changes were not marked and additional data would be needed in order to confirm if a significant decrease in the activity of these molecules occurs. Surprisingly an increase in MMP9 activity was observed in animals treated with PD166793. This may be due to the fact that MMP9 activity is only minimally affected by PD166793. It could also indicate that MMP9 as a different and/or more complex role in the development of lung metastasis.

Knowing that MMPs play a role in metastasis development is important, however, it is also fundamental to understand what are the mechanisms by which the inhibition of MMPs causes a phenotypic decrease in metastatic behavior. In addition to the degradation of the extracellular matrix, MMPs are thought to have other roles in cancer development. MMPs stimulate angiogenesis, cancer cell proliferation and may have a role in inhibition of apoptosis. We intended to further characterize cell proliferation, cell death and stroma development in our system.

MMP expression has been correlated with high proliferative indexes in cancer. MMPs are induced by growth factors and the inhibition of MMPs via TIMPs causes decrease in in vivo tumor proliferation [3]. To evaluate cell proliferation we scored from 1

to 7 the mitotic index and nuclear pleomorphism [97]. We found that there was significantly higher mitotic index and increase nuclear pleomorphism in non PD166793-treated animals. These two parameters are clear indicators of tumor malignancy that is related with highly proliferative, invasive and metastatic characteristics [97]. We confirmed this evaluation by calculating the tumor cell PCNA proliferative index. We observed that PCNA indexes were higher on lung metastasis originated from non PD166793-treated animals. Additionally, indexes superior to 30% are considered indicators of high proliferation [112]. Taken together these results suggest that inhibition of MMPs is associated with decrease in cell proliferation.

Additionally, MMPs can be related to cancer cell death. MMPs cause the loss of contact from neoplastic cells to the basement membrane and are responsible for anoikis a special type of apoptosis [3]. Furthermore, there is an increase in apoptosis in genetically manipulated MMP3 transgenic mice [3]. Cell death, may it be by apoptosis or necrosis, is a very important tumor characteristic. Highly malignant tumors (that are more likely to metastasize) have usually extensive cell death. In here we evaluated cell death histologically. We attributed a score from one to seven in accordance with the extension of cell death and we observed that treatment with PD166973 did not increase the amount of cell death. To confirm this finding we used protein extracts from tumors in order to perform WB analysis of cleaved-caspase 3. Caspase 3 is one of the main molecules involved in apoptosis and it is known to activate in both intrinsic and extrinsic apoptotic pathways. Furthermore caspase 3 has been reported to be a good general indicator of the apoptotic status [96]. There was no significant difference in the expression of cleaved-

caspase3 protein in samples collected from non-PD166793 treated animals. TUNEL assay did not revealed a significant difference between treated and non-treated animals. These results suggest that MMP inhibition by PD166793 may not play a considerable role in cancer cell death in our system.

Finally we studied the variation of stroma development in the metastatic foci in different conditions of MMP activity. BC tumor cell lines transcribe, synthesize and secrete MMP2, MMP3, and MMP9. The production of these MMPs is related with the extra-cellular matrix, fibroblast and collagen fibers that surround the tumor cells [10]. Stroma cells fibroblasts are mediators and stimulators of the production of MMPs in tumor cells [2]. Additionally, most MMPs are localized in the tumor stroma. MMP2, and MMP3 are observed in a widespread manner in peritumoral fibroblasts, and MMP9 is observed in focal endothelial cells [35]. To evaluate differences in fibrous stroma in the metastatic neoplastic foci we quantified the amount of stroma by histological evaluation. The results were confirmed the evaluation by vimentin immunostaining, a marker for mesenchymal differentiation characteristic of stromal fibroblasts. We observed that tumor stroma was more prominent in non-PD166793 treated animals. This suggests that MMPs may play a role development of tumor stroma may and/or vice versa.

In conclusion we observed a significant decrease in breast cancer lung metastasis in a rodent model associated with selective inhibition of MMPs by PD166793. In addition we observed significant decrease in cell proliferation, and stroma development in animals that were treated with PD166793. These results suggest that in addition to inhibition of

extracellular matrix degradation, MMPs play a role in cancer cell proliferation that may be conditioned by tumor stroma development.

## **CHAPTER V**

### **EFFECTS OF MMP2 INHIBITION BY TIMP2 IN THE DEVELOPMENT OF BREAST LUNG METASTASIS IN A RAT MODEL**

#### **Introduction**

The development of metastatic tumor cells depends on multiple factors including tumor environment [80] and molecular and genetic characteristics of neoplastic cells [R42]. MMPs have been extensively associated with cancer development [113, 114, 115, 116, 117, 118, 119, 120, 123, 124, 125]. We aim to study the role played by matrix metalloproteinase 2 (MMP2) and its physiological inhibitor (TIMP2- Tissue inhibitor of MMP2) in the development of BC lung metastasis.

MMP2 belongs to a broad family of zinc-dependent proteinases that play a key role in extracellular matrix degradation, and are implicated in numerous pathogenic processes [126, 127, 128]. Tumor cells are believed to secrete MMP2. In addition to the degradation of the extracellular matrix (ECM), MMP2 and TIMP2 are suspected of having other roles in cancer development such as inhibition of apoptosis [6], and regulation of cell proliferation [3]. High serum concentration and over-expression of MMP2 in breast cancer patients, are related to poor prognosis, decreased survival, increased tumor size, increased invasiveness and metastatic behavior [18]. Data from experimental animal models also correlates MMP2 expression with invasive behavior and increased metastasis [R68].

Transfection with MMP2 in breast cancer cells increases brain, liver, bone and kidney metastasis but does not significantly affect lung metastasis [10].

TIMPs are specific inhibitors of matrixins that control MMPs activities [4]. Four homologous TIMPs have so far been characterized: TIMP1 through -4. They regulate MMPs activity controlling in this way the breakdown of extracellular matrix components important for invasion and metastasis [129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139]. An increase in the amount of TIMPs relative to MMPs may function to decrease MMP activity and block tumor cell invasion and metastasis, if this protease activity is a critical determinant for these processes. In fact, tumor cell invasion and metastasis can be inhibited by up-regulation of TIMP expression or by an exogenous supply of TIMPs [54, 56].

At first, TIMP2 was believed to suppress invasion and metastasis. However, the complexity of TIMP2 /MMP interactions led to reconsideration of the role of TIMP2 in cancer [4]. TIMP2 expression in BC patients has been correlated with advanced disease, decreased survival time, increases in tumor size, node-positive status and tumor recurrence [140]. Paradoxically, the genetic manipulation of cancer cells has correlated experimental TIMP2 overexpression with decreased metastatic behavior; cells genetically modified to overexpress TIMP2 have fewer, smaller and less invasive *in vivo* characteristics [49, 52].

Establishment of metastasis in the lung and development of metastasis originated from pulmonary neoplasms have been connected to MMP2 [105, 110] and TIMPs [98]. Because pulmonary metastases are a common event in breast cancer clinical outcome, several MMP inhibitor drugs have been tested in pulmonary breast cancer metastasis [106].

Here, we intend to determine if inhibition of MMP2 is important in the development of pulmonary metastasis of breast cancer in a rodent syngeneic model. To do this we over expressed TIMP2 in ENU1564 cells, an ethyl-nitrosourea-induced rat adenocarcinoma cell line. We will further characterize the metastatic system, evaluating if MMPs influence cancer cell proliferation, cancer cell death and tumor stroma development.

## **Materials and methods**

### *Tumor cell line*

The ENU1564 tumor cell line used in this study was developed in our laboratory and originated from an N-ethyl-N nitrosourea-induced mammary adenocarcinoma in a female Berlin-Druckrey IV (BD-IV) rat. This cell line is highly metastatic to lung, brain and bone tissues [R142].

### *Rat inoculation*

Forty-day-old BD-IV rats were used. The animals were obtained from a colony maintained at Texas A&M University in accordance with institutional animal care guidelines. The syngeneic animals were inoculated with  $1 \times 10^4$  tumor cells in the left ventricle. Inoculation was performed on animals under Ketamine (87mg/kg, intramuscular injection) anesthesia. The animals were humanely euthanatized using Pentobarbital

(150mg/kg, intraperitoneal injection) when showing clinical signs of discomfort. Complete necropsies were performed and tissues were sampled for histology evaluation.

### *Transfection experiment*

Dr Stetler-Stevenson, (NIH, USA) graciously provided the TIMP2 human plasmid [R12]. TIMP2 human sequence has high homology with the respective TIMP2 rat sequence (BLAST). pcDNA-TIMP2 was generated by sub-cloning TIMP2 cDNA into the pcDNA3.1 vector (Invitrogen, Carlsbad, CA). TIMP2 cDNA was released by cutting with Pst1 and Apa1 restriction enzymes, gel purified, blunt-ended ligated into the Xba1 restriction site PcDNA 3.1. The orientation of cDNA was verified by restriction enzyme digestion and sequencing. The vector has a eukaryotic selection marker (neomycin resistance gene) that allows selection under G418 (Invitrogen). After linearization with Nru1, the plasmid was transfected into ENU1564 cells using Lipofectamine2000 (Invitrogen). After transfection, the cells were placed in antibiotic selective media (G418) to select clones that had successfully been transfected with the plasmid. The colonies were screened by WB for the expression of TIMP2 protein. Animals were inoculated with the stably transfected ENU1564-TIMP2 cell line. The animal inoculation was done in groups of nine animals. Nine control animals were inoculated with ENU1564 cells at the same time.



### *Immunohistochemistry (ICH)*

Five-micron (5  $\mu$ m) paraffin-embedded sections and 12 $\mu$ m frozen sections were used. Deparaffinization, rehydration and antigen-retrieval were done by immersion of slides in DECLERE® (Cell Marque, Hot Springs, AR) commercially available buffer in moist heat (pressure cooker) for 15 minutes. Potential non-specific binding sites were blocked with 5% normal goat or rabbit serum in PBS. After blocking, the sections were incubated with primary antibodies in dilutions of 1:200 for MMP2; 1:25 for MMP3 and -9 purchased from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA); 1:500 for PCNA and 1:300 for Vimentin (antibodies for PCNA and vimentin were purchased from DAKO, Dako Cytomation, Denmark). After three five-minute washes in PBS, the sections were then incubated with biotin-conjugated anti-rabbit and anti-goat IgG (Vector Laboratories, Burlingame, CA). A Vector-ABC streptavidin-peroxidase kit with a benzidine substrate was used for color development. Counter-staining was done with diluted hematoxylin. Sections that were not incubated with primary antibody served as negative control.

### *Western blotting*

The tissue was homogenized in lysis buffer in a 1V:10V dilution (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 1 mM EDTA, pH 8.0, and 0.1% SDS), supplemented with a mixture of protease inhibitors. Samples were run on a 9-12% SDS polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated one hour in blocking buffer (20 mM Tris-HCl buffered saline containing 5%

nonfat milk powder and 0.1% Tween 20). Blots were incubated at 4°C overnight with anti-MMP2 (1:2000 (antibody from Santa Cruz Biotech, Santa Cruz, CA), cleaved-caspase 3 (1:1000) (antibody from Cell Signaling, Beverly, MA) and washed extensively and then incubated for one hour with a 1:5000 dilution of anti-primary antibody. After additional washes, the blots were incubated with chemiluminescent substrate, according the directions in the kit (SuperSignal® West Pico, Pierce, Rockford, IL).

#### *PCNA index evaluation*

The evaluation of PCNA index was performed according to the literature [L17]. After IHC staining with PCNA antibody the number of cells with positive nuclear staining was evaluated in a total of 500 cells in five different high power fields (400X).

#### *Statistical analysis*

Student t-test was performed to evaluate all parameters including densitometry values obtained from Western blotting-autoradiographs analysis using Fluor S MultiImager® technology from Biorad (Hercules, CA). Differences were considered statistically significant when  $p \leq 0.1$ .

## Results

### *MMP expression in lung metastasis*

We evaluated the expression of MMP2 in lung metastasis using IHC. We observed that neoplastic cells have strong expression of these molecules diffusely in the cytoplasm (See chapter IV Figure1). To confirm these results we performed WB in lung metastatic tissue. We observed no significant difference in the levels of MMP2 in lung metastasis when compared with normal lung (See chapter IV Figure2).

### *Transfection experiment*

To determine if TIMP2 overexpression and MMP inhibition had a effect in the development of BC lung metastasis we inoculated ENU1564-TIMP2 cells in the left ventricle and compared with animals inoculated with ENU 1546 control cells. We observed that the lung weight/body weight and lung weight/brain weight ratios were higher in animals inoculated with ENU1564 cells compared with animals inoculated with ENU1564-TIMP2 ( $p \leq 0.021$  and  $p \leq 0.128$  respectively). There were higher numbers and larger numbers of lung metastasis in animals inoculated with ENU1564 cells compared with animals inoculated with ENU1564-TIMP2 ( $p \leq 2.7E-06$  and  $p \leq 7.08E-7$ , respectively) (Table 6) (Figures 42, 43, 44, 45 & 46).

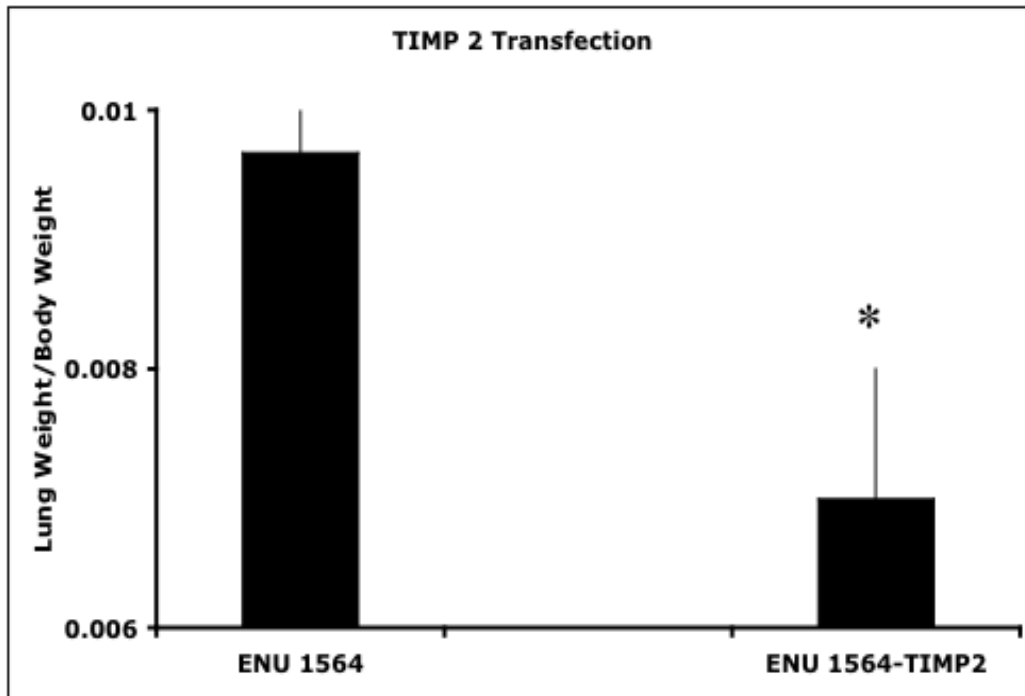


Figure 42. Effects of TIMP2 overexpression in the development of lung metastasis. Evaluation of lung weight/body weight ratio.(\*) for statistically significant when  $p \leq 0.05$ .

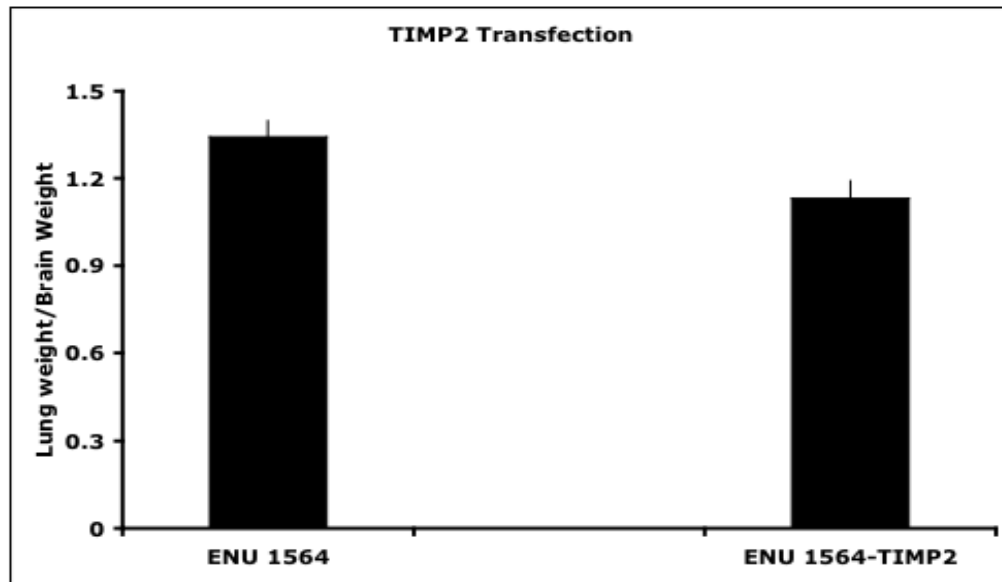


Figure 43 Effects of TIMP2 overexpression in the development of lung metastasis.  
Evaluation of lung weight/body weight ratio.

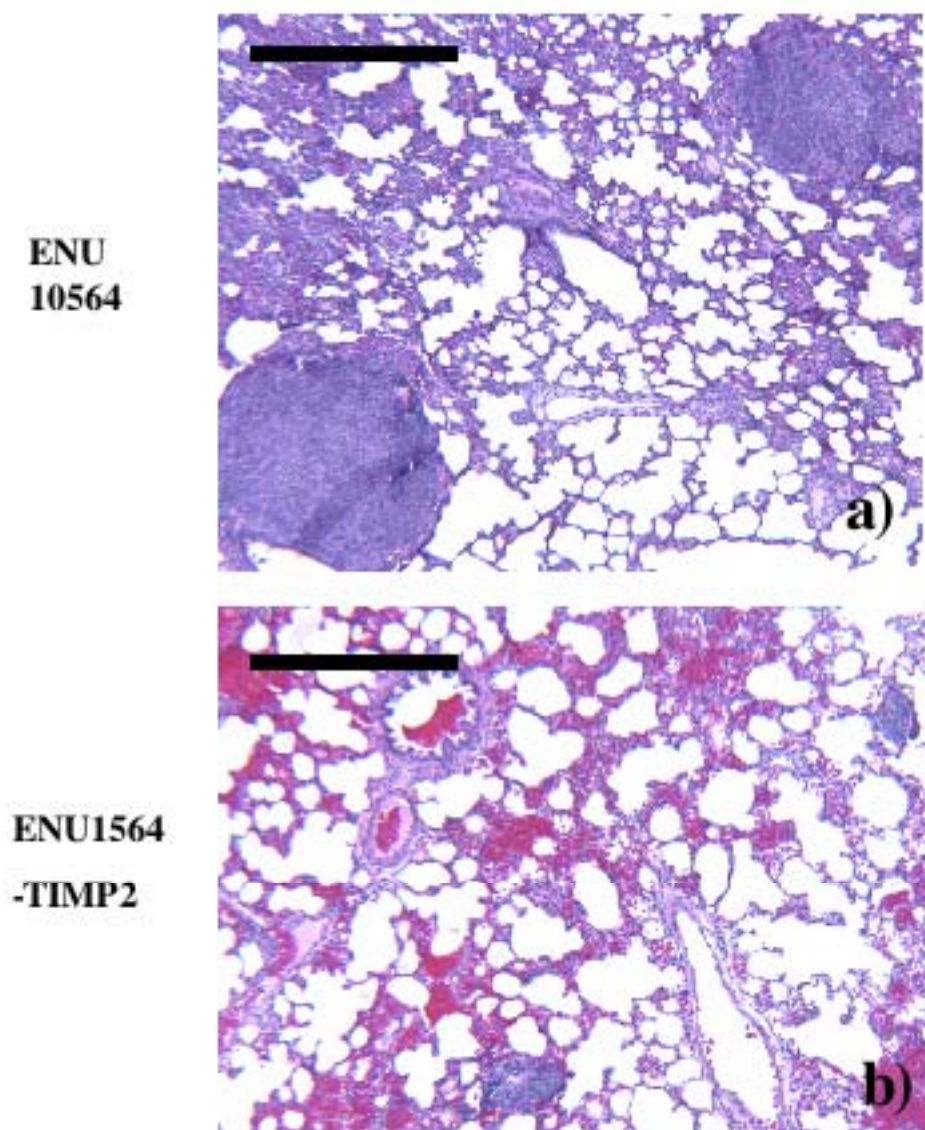


Figure 44. Effects of TIMP2 overexpression in the development of lung metastasis. Histological evaluation of pulmonary metastasis size and number in animals inoculated with ENU 1564(a) and (b) animals inoculated with ENU1564-TIMP2. Bars indicate 100 $\mu$ m.

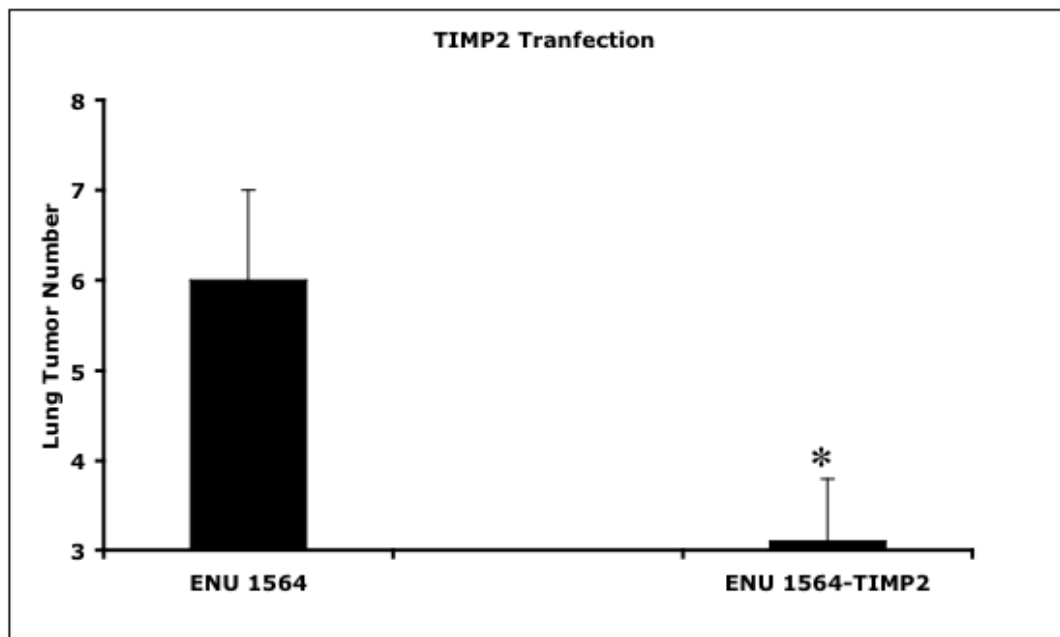


Figure 45. Effects of TIMP2 overexpression in the development of lung metastasis. Evaluation of tumor size variation between animals in animals inoculated with ENU 1564 and animals inoculated with ENU1564-TIMP2. (\*) for statistically significant when  $p \leq 0.05$ .

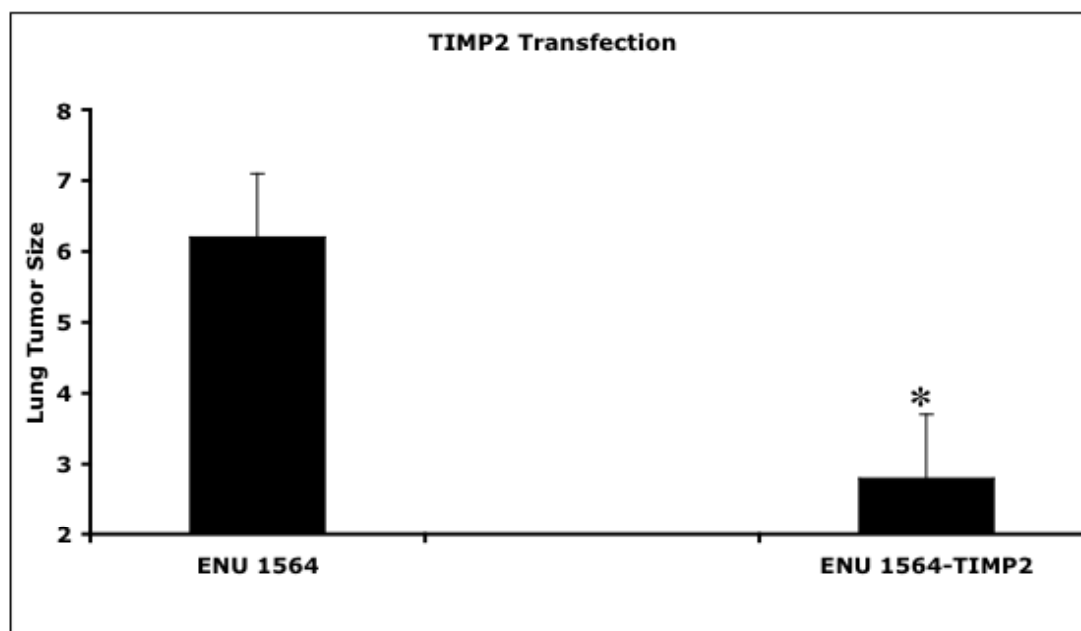


Figure 46. Effects of TIMP2 overexpression in the development of lung metastasis. Evaluation tumor number variation between animals in animals inoculated with ENU 1564 and animals inoculated with ENU1564-TIMP2. (\*) for statistically significant when  $p \leq 0.05$ .



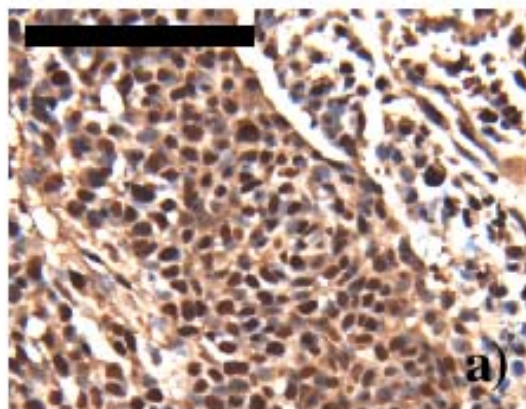
**Table 6.** Effect of TIMP2 overexpression in lung metastasis development.

	ENU1564 - TIMP2	ENU1564	p value
Mitotic index	3.22±1.39	7±0	1.94E-5
Nuclear pleomorphism	3±1.11	6.33± 0.70	1.63E-6
Stroma abundance	3.22±1.39	6.33±0.86	3.35E-5
Cell death	3.22±1.48	6.11±1.16	0.0001
PCNA index	24.6%	43.5%	
Number of tumors	3.11±0.78	6±1	2.7E-6
Tumor size	2.8±0.92	6.22±0.97	7.08E-7

#### *Evaluation of cell proliferation*

In order to determine if there was an effect of MMP2 inhibition and TIMP2 overexpression in tumor cell proliferation we evaluated histologically the mitotic index and nuclear pleomorphism. We confirmed these results by calculating the PCNA proliferative index. There was significantly higher mitotic index in animals inoculated with ENU1564 cells ( $p \leq 1.94E-5$ ). There was also increase nuclear pleomorphism in animals inoculated with ENU1564 cells ( $p \leq 1.6E-6$ ). Additionally, we observed that PCNA indexes were higher on lung metastasis originated from animals inoculated with non-transfected cells (43.5%) when compared with animals inoculated with ENU1564-TIMP2 cells (24.6%) (**Figure 47**).

**PCNA**  
**ENU 1564**



**PCNA**  
**ENU 1564-**  
**TIMP2**

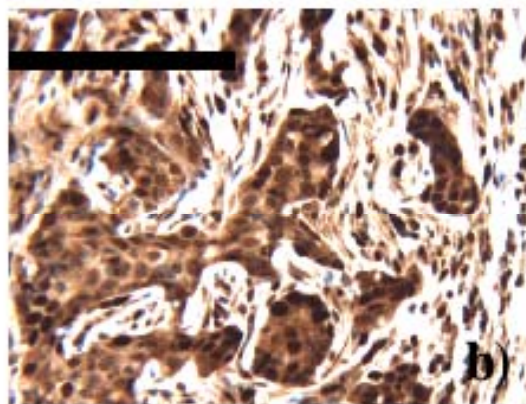


Figure 47. Effects of TIMP2 overexpression in tumor cell proliferative index. Immunohistochemical staining (brown) of PCNA in the nucleus of neoplastic cells in lung metastatic foci in animals inoculated with ENU 1564(a) and (b) animals inoculated with ENU1564-TIMP2. Bars indicate 100 $\mu$ m.

### *Evaluation of cell death*

To evaluate differences in cell death in the metastatic neoplastic foci we quantified the cell death phenotype by histological evaluation and confirmed the evaluation by immunoblotting for cleaved-caspase 3. We observed that there was a higher amount of cell death in animals inoculated with non-transfected cells ( $p \leq 0.00017$ ). There was also higher expression of cleaved-caspase3 protein in samples collected from animals inoculated with non-transfected cells ( $p \leq 0.1$ ) (**Figure 48**).

### *Evaluation of metastatic foci stroma*

To evaluate differences in fibrous stroma in the metastatic neoplastic foci we quantified the amount of stroma by histological evaluation and confirmed these results by Vimentin IHC staining. We observed that there was more prominent stroma in animals inoculated with ENU1564 cells when compared with animals inoculated with ENU1564-TIMP2 cells ( $p \leq 3.5E-5$ ) (**Figure 49&50**).

## **Discussion**

In the context of BC prognosis, MMP2 is thought to play an important role in breast cancer invasion and metastasis [R44]. The evaluation of MMP2 expression in lung metastasis using IHC (**chapter IV**) revealed strong expression of this molecule diffusely in

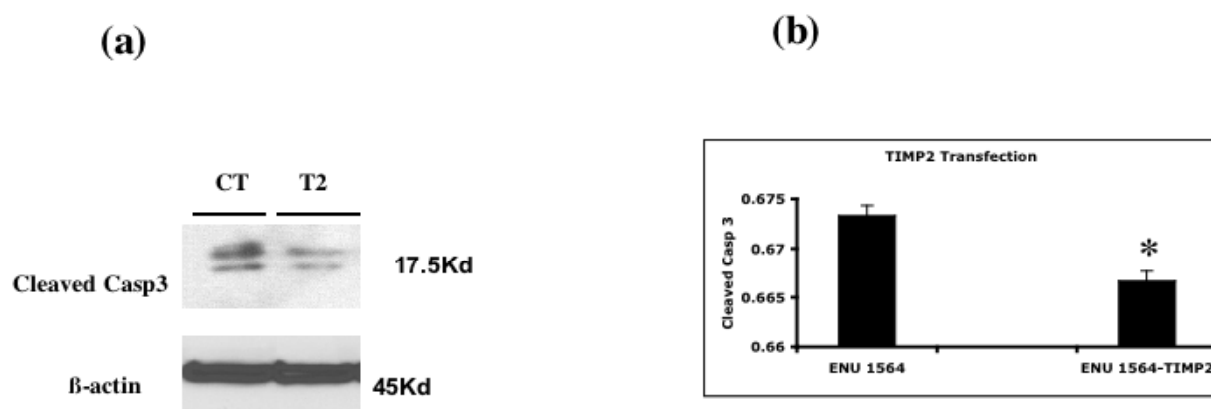


Figure 48. Effects of TIMP2 overexpression in lung metastasis apoptosis. (a) Expression of cleaved-caspase 3 in lung metastatic foci in animals inoculated with ENU 1564 vs animals inoculated with ENU1564-TIMP2. The membranes were striped and re-probed with  $\beta$ -actin antibody to confirm equal loading (b) Quantitative analysis of cleaved-caspase3 was determined by densitometry. The results shown in the histogram are the mean  $\pm$  standard deviation from three control and three tumor samples. (\*) for statistically significant when  $p \leq 0.1$ .

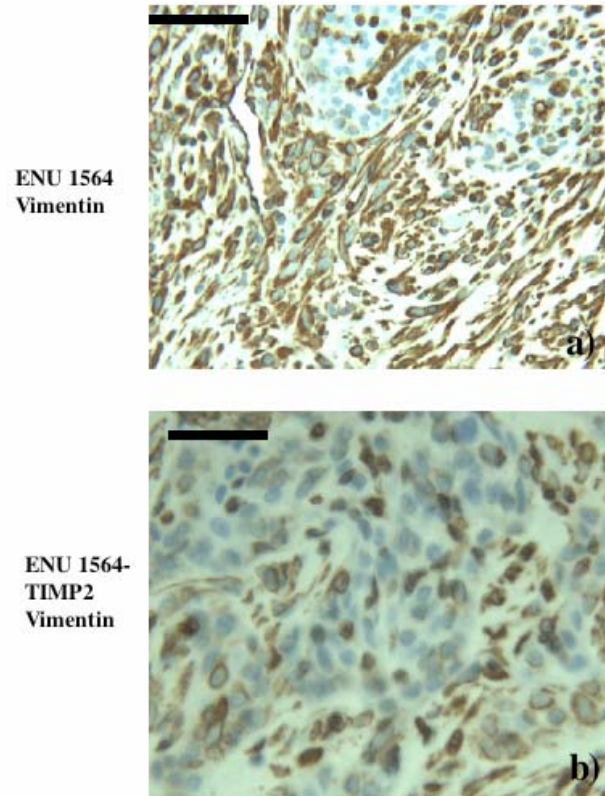


Figure 49. Effects of TIMP2 overexpression in tumor stroma development. Immunohistochemical staining (brown) of vimentin in the cytoplasm of stromal fibroblasts in lung metastatic foci of animals inoculated with ENU 1564(a) and (b) animals inoculated with ENU1564-TIMP2. Bars indicate 100 $\mu$ m.

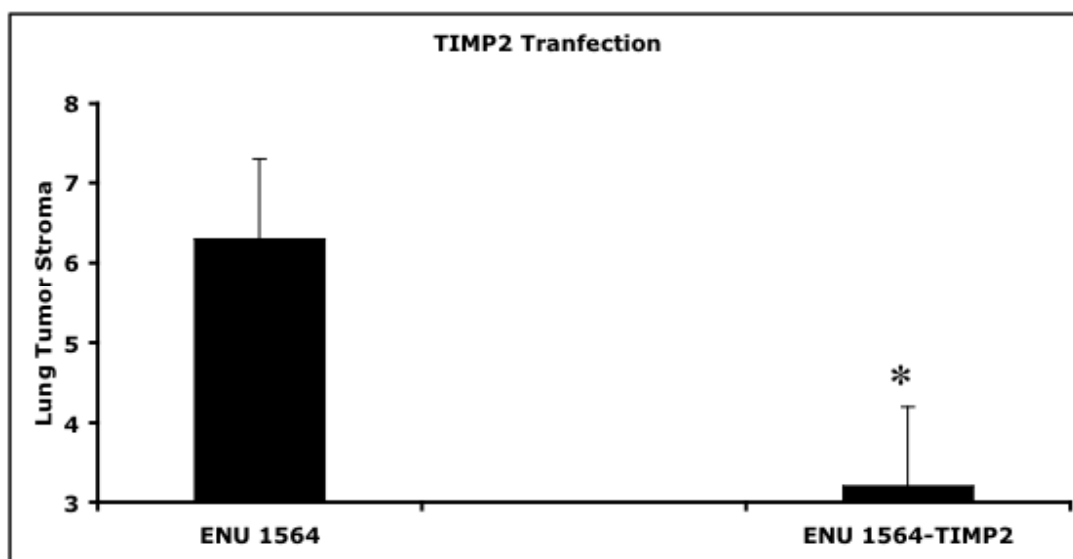


Figure 50 Effects of TIMP2 overexpression in the development of lung metastasis. Evaluation of tumor stroma variation between animals inoculated with ENU 1564 and ENU1564-TIMP2.

the cytoplasm of neoplastic cells. Because of these results and to determine if MMP2 plays a role in the development of metastasis we decided to inhibit MMP2 expression by overexpression of its physiological inhibitor -TIMP2.

We generated a cell line over expressing TIMP2 (ENU1564-TIMP2). Despite the fact that TIMP2 cancer expression is associated with poor prognosis [129, 130, 132, 133], transfection with TIMPs in in vivo models decreases not only tumor growth but also metastatic behavior [53]. The reason for this paradox as not yet been clarified and clearly

more studies are needed in order to understand the complex mechanisms of TIMP2/MMP2 interaction. The fact that together with MT-MMP1, TIMP2 participates in the activation of MMP2 [4] would explain why it has been related with bad prognosis. However, experimental TIMP2 overexpression is related with decrease node and pulmonary metastasis in bladder cancer [52]; adTIMP2 delivery decreases the numbers of pulmonary metastasis [49]; and 91 These results clearly emphasize the role of TIMP2 in metastatic development and introduce TIMP2 as a possible therapeutical agent. We hypothesize that TIMP2 is important to the development of pulmonary metastasis. As in agreement with the results obtained in the drug experiment (**see chapter IV**), we observed that the lung weight/body weight and lung weight/brain weight ratios were higher in animals inoculated with ENU1564 cells compared with in animals inoculated with ENU1564-TIMP2 cells and that there are higher and larger numbers of lung metastasis in animals inoculated with non-transfected cancer cells. Once again these results are supportive of the premise that MMPs are important and play an active role in the pulmonary metastatic cascade of breast cancer. Because TIMP2 overexpression causes decrease in MMP2 activity in orthotopic tumors (**see chapter III**) we can suggest that MMP2 is also involved in the development of lung metastasis in our model.

Knowing that TIMP2/MMP2 play a role in metastasis development is important, however, it is also fundamental to understand what are the mechanisms by which the inhibition of MMP2 causes a phenotypic decrease in metastatic behavior. In addition to the degradation of the extracellular matrix, MMP2 and TIMP2 are thought to have other roles in cancer development.

MMP2 has been associated with breast cancer malignant characteristics such as high proliferation of cancer cells. By causing degradation of the extra cellular matrix MMP2 is indirectly responsible for growth factor release that causes increased tumor cell proliferation [3]. At the same time TIMP2 by regulating MMP2 activity may influence tumor cell proliferation. In addition to inhibiting tumor cell invasion and metastasis, overexpression of TIMPs in tumor cells may also inhibit primary tumor growth. To evaluate cell proliferation we scored from 1 to 7 the mitotic index and nuclear pleomorphism [97]. We found that there was significantly higher mitotic index and increase nuclear pleomorphism in animals inoculated with ENU 1564 cells when compared with ENU1564-TIMP2 cells. These two parameters are clear indicators of tumor malignancy that is related with highly proliferative, invasive and metastatic characteristics [97]. We confirmed this evaluation by calculating the tumor cell PCNA proliferative index. We observed that PCNA indexes were higher on lung metastasis originated from animals inoculated with ENU 1564 cells when compared with ENU1564-TIMP2 cells. Additionally, indexes superior to 30% are considered indicators of high proliferation [96]. Taken together these results suggest that inhibition of MMP2 and TIMP2 overexpression are associated with decrease in cell proliferation.

Tumor cells can undergo cell death by apoptosis or necrosis. There are indications that MMPs can cause loss of contact from neoplastic cells to the basement membrane and are responsible for anoikis a special type of apoptosis [3]. TIMP2 is said to decrease apoptosis [6]. TIMP2 has anti-apoptotic effect in epithelial cells and TIMP4 has anti-apoptotic activity in BC cells [43]. We evaluated cell death by histological examination.



We attributed a score from one to seven in accordance with the extension of cell death. We observed that there was a higher amount of cell death in animals inoculated with ENU 1564 cells when compared with ENU1564-TIMP2 cells. To confirm this finding we used protein extracts from tumors to perform WB analysis of cleaved-caspase 3. Cleavage of caspase 3 is one of the main events of both intrinsic and extrinsic apoptotic pathways. Cleaved caspase 3 has also been reported to be a good indicator of the apoptotic status [R110]. We observed higher expression of cleaved-caspase3 protein in samples collected from animals inoculated with ENU1564 cells when compared with ENU1564-TIMP2 cells. These results suggest that there may be a role of MMP2 inhibition and TIMP2 activity in cancer cell death.

Finally we studied the variation of stroma development in the metastatic foci in different conditions of MMP2 activity. Within breast cancer tumor foci, both fibroblastic stromal cells and neoplastic epithelial cells can express and secrete MMP2 [10, 35]. The exact role and contribution of these different tumor cells in metastasis and tumor invasion is still the object of intense scrutiny. Stromal fibroblasts are thought to interact with cancerigenous epithelial cells and act as mediators and stimulators of the production of MMPs in tumor cells [2]. TIMP-2 expression may influence the stromal response to tumor invasion, indicative of aggressive behavior in a subset of breast carcinomas. To evaluate if TIMP2 overexpression and decrease in MMP2 activity causes differences in fibrous stroma development in the metastatic neoplastic foci we quantified the amount of fibrous stroma by histological evaluation. The results were confirmed the evaluation of vimentin immunostaining, a marker for mesenchymal differentiation characteristic of stromal

fibroblasts. We observed that animals inoculated with ENU1564 cells developed more prominent tumor fibrous stroma when compared with animals inoculated with ENU1564-TIMP2 cells. This suggests that there may be a relation between tumor stroma development and MMP2 activity.

In conclusion we observed a significant decrease in breast cancer lung metastasis in a rodent model associated with TIMP2 overexpression in breast cancer cells. In addition we observed significant decrease in cell proliferation, stroma development and cell death in animals that were inoculated with ENU1564-TIMP2. These results suggest that in addition to decreasing development of BC lung metastasis, MMP2 and TIMP2 play a role in cancer cell proliferation and cell death.

## CHAPTER VI

### SUMMARY/CONCLUSIONS

This dissertation provides a comprehensive analysis of the role of matrix metalloproteinases 2, 3 and 9 in the development of brain and lung metastatic disease of breast cancer. Additionally it explores the potential of a rat syngeneic model for breast cancer in the study of molecular genetics of distant breast cancer metastasis.

Chapter I provides a introductory comprehensive review of MMP biology, their role in (breast) cancer development, and in neurological disease.

Chapter II contains the first manuscript generated from this work that is currently in print in *Clinical & Experimental Metastasis*. This chapter describes the expression of MMP2, -3 and -9 in breast cancer brain metastasis at the level of protein and mRNA expression as well as the activity of MMP2 and MMP3 in the brain metastatic foci. Additionally the importance of these molecules in the development of brain metastatic disease is underline by inhibition of metastatic disease observed with treatment with a specific MMP inhibitor-PD166793.

Chapter III includes the second manuscript generated from this work that is currently under review by *Breast Cancer Research*. This chapter focuses on the role of MMP2 in breast cancer brain metastasis. This proteinase is inhibited by overexpression of its physiological inhibitor, TIMP2. A decrease of MMP2 activity caused inhibition of brain metastasis development. Data presented on this chapter strongly suggests that the

mechanism of MMP2 up regulation in breast cancer brain metastasis is associated with astrocytic factors and is mediated, at least in part by the ERK1/2 pathway.

Chapters IV and V describe the role of MMPs in the development of breast cancer pulmonary metastasis. Inhibition of MMP activity is accomplished by the use of PD166793 (**chapter IV**) and TIMP2 overexpression (**chapter V**). Decrease in lung metastatic disease was observed with both approaches. Histological evaluation of these different conditions of MMP activity was conducted in both cases in order to elucidate additional factors important in MMP activity. TIMP2 overexpression and treatment with PD166793 cause decrease in tumor cell proliferation and are associated with less prominent tumor stroma development. Additionally, decrease in MMP2 activity and TIMP2 overexpression appear to decrease the development of intratumoral apoptosis.

In conclusion, this work provides an analysis of the role of MMPs in the development of cancer metastatic disease in particular to the MMPs in breast cancer brain metastatic disease.

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